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PRINCIPAL INVESTIGATOR: Joseph C. Glorioso, Ph.D.

RECIPIENT ORGANIZATION: University of Pittsburgh
Pittsburgh, Pennsylvania 15260

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FOREWORD

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I. Introduction

Parkinson's disease (PD) is a neurodegenerative condition in which degeneration of dopaminergic neurons in the substantia nigra results in a clinical syndrome of rigidity, bradykinesia and tremor. The cause of the neurodegeneration is not known, but substantial evidence derived from a variety of model systems suggested that anti-apoptotic peptides such as bcl-2, or neurotrophic factors such as glial-derived neurotrophic factor (GDNF) might show or prevent the death of the dopaminergic cells. In this project we have proposed to develop and test genomic Herpes Simplex virus (HSV)-based vectors that deliver and express bcl-2, GDNF, or both in a well-characterized rodent model of Parkinson's disease.

II. Body

Substantial progress has been made on all three tasks outlined in the Statement of Work, with modifications as dictated by the interim experimental findings. The three tasks were:

- A. Construction of latency vectors altered in the latency promoter region.
- B. Construction of an HCMV GDNF vector for short-term studies of neuron survival.
- C. Test the ability of the SCMV-bcl-2 vector to block apoptosis cell death in a rodent model of PD.

A description of the accomplishments regarding each task are described below.

- A. Construction of latency vectors altered in the latency promoter region.

The goal of this work was to engineer a promoter that was capable of higher level long-term expression. To accomplish this goal we examined two approaches. First, we engineered a series of promoter chimeras in which the HSV latency promoters were juxtaposed to the strong constitutive HCMV IE promoter (Fig.1). The promoter chimeras were linked to the lacZ transgene and inserted into the UL41 gene locus of a replication defective vector deleted for both the ICP4 and ICP27 IE genes. LacZ was selected over EGFP as the transgene of choice due to the fact that we had previously generated recombinants with lacZ reporter gene in the UL41 gene locus for use as isogenic controls (Krisky et al., 1997; Krisky et al., 1998) and because of the high background levels of EGFP frequently observed in sections from many tissues. The second approach was based on a report by Lachmann and Efstathiou 1997, in which they introduced an internal ribosome entry site (IRES) upstream of the lacZ transgene when the expression cassette was inserted within the LAT intron far downstream of the LAPs. The addition of the IRES in their constructs resulted in higher transgene levels. Thus, we engineered an additional series of chimeric constructs with an IRES juxtaposed between the LAPs and the lacZ (Fig.2).

The chimeric promoter recombinants were used to transduce primary dorsal root ganglia (DRG) cultures and expression of the lacZ transgene was observed over a period of 28 days. The HCMV IE promoter alone (Fig.3A) was transiently active. As we have previously observed (Goins et al., 1994; Goins et al., 1999; Wolfe et al., 1999), the LAP2 promoter was not active early (2d) but began to express the transgene by 7 days (Fig.3B) and remained active out to 28 days although the level of expression was not substantial. When the LAP2 promoter was juxtaposed to the HCMV IE promoter either in the presence (Fig.3D) or absence (Fig.3C) of LAP1 high-level long-term transgene expression was observed. These vectors continued to express at this level out to one month, the longest time point examined in this study. We are currently evaluating these vectors in primary cortical neuronal cultures as well as in *in vivo* studies in rat CNS. Future experiments will determine which elements within LAP2 are responsible for this activity and will examine whether this ability is dependent on position or orientation. We are currently constructing vectors with the LAP2-HCMV hybrid promoter in which the lacZ transgene is replaced by GDNF for testing in the 6-OHDA model of Parkinson's.

Addition of the IRES immediately downstream of the LAPs in our recombinants did not alter the level or duration of transgene expression. This result was not entirely unexpected, since in the constructs of Lachmann and Efstathiou the expression cassette was inserted far downstream from LAP within the LAT intron. Multiple stop codons are located within the LAT intron and this region is capable of forming complicated stem-loop RNA secondary structure that could interfere with the expression of sequences encoding proteins. However, in our constructs the transgene is inserted immediately downstream from the LAPs and do not contain any of the LAT intron sequences that could interfere with translation of the inserted transgene.

We are currently finishing the construction of the LAP1-Brn recombinant viruses and are in the process of examining Brn expression in rat CNS and PNS using *in situ* hybridization studies with probes specific for the different Brn family members

B. Construction of an SCMV GDNF vector for short term studies of neuron survival.

We have recently engineered a replication defective vector deleted for the ICP4 in which we have inserted an HCMV IEp-GDNF expression cassette into the UL41 gene locus (Fig.4A) to achieve high-level transient expression of the neurotrophin. The structure of the GDNF expression vector was confirmed by Southern blot analysis (Fig.4B) prior to use in both *in vitro* and *in vivo* studies. Expression of the neurotrophin was examined in Vero cell cultures following infection with either the GDNF expressing vector (DHG) or the control vector that expresses lacZ (DOZ.1). High levels of GDNF were observed by ELISA both in the cell lysate and released into the culture medium following DHG

infection (Fig.4C). Stocks of this recombinant have now been gradient purified and are being tested in the 6-OHDA model.

- D. Test the ability of the SCMV:bcl2 vector to block apoptotic cell death in a rodent model of PD.

This aim has been accomplished and the results reported in an article published in PNAS (enclosed). Because of difficulty establishing the MPTP model in the mouse in our laboratory, we proceeded to test the effectiveness of the vector using the well-characterized 6-hydroxydipamine (6-OHDA) model in the rat [Sauer, 1994 #1085]. We employed an HSV vector deleted for the IE genes ICP4, ICP22, and ICP27, with the SCMV IE promoter driving bcl-2 expression in a cassette inserted in the tk locus of the vector genome. Transfection of primary cortical neurons in culture with the bcl-2 producing vector protected those cells from naturally-occurring apoptotic cell death over 3 weeks. The expression of bcl-2 vector-injected substantia nigra was demonstrated by RT-PCR using primers specific for the human bcl-2 transcript.

Injection of the bcl-2 producing vector into the substantia nigra of rats 1 week before injection of 6-OHDA into the ipsilateral striatum increased the survival of neurons in the SN, detected by retrograde labeling of those cells with fluorogold. The vector also preserved the neurotransmitter phenotype of the transfected cells, measured by the tyrosine hydroxylase (TH) immunoreactivity of the lesioned-vector injected SN. Approximately twice as many cells of the SN, labeled retrogradely by FG or identified by TH-immunoreactivity.

Using the same vector, we examined the protection of SN cells from apoptotic cell death and the preservation of neurotransmitter phenotype when the 6-OHDA was injected into striatum 4 weeks after injection of the vector into the SN, and the animals sacrificed 2 weeks after 6-OHDA as in the previous experiment (7 weeks after vector injection). No protection was demonstrated at that time-point, either by measures of cell survival (fluorogold labeling) or TH immunoreactivity. This result was anticipated because of our previous data regarding the time course of expression employing the CMV IEp to drive transgene expression from the vector in other model systems. We are proceeding now to construct the LAP2 vector as previously proposed for year 2 of the project.

III. Key Research Accomplishments

- LAP2-HCMV hybrid promoter yields high-level long-term transgene expression in primary DRG cultures.
- Construction of the HCMV:GDNF HSV vector.

- Demonstration that the SCMV:bcl-2 vector protects dopaminergic neurons in the substantia nigra from apoptotic cell death induced by the neurotoxin 6-OHDA.
- Demonstration that the SCMV:bcl-2 vector preserves the neurotransmitter phenotype of dopaminergic neurons in the substantia nigra following 6-OHDA lesioning.

IV. Reportable Outcomes

Manuscripts:

1. Wolfe D, Goins WF, Yamada M, Moriuchi, S, Krisky DM, Oligino TJ, Marconi PC, Fink DJ, Glorioso JC. Engineering Herpes Simplex Virus Vectors for CNS Applications. *Exp Neurol* 159:34-46, 1999.
2. Yamada M, Oligino T, Mata M, Goss JR, Glorioso JC, Fink, DJ. Herpes Simplex Virus Vector-Mediated Expression of Bcl-2 prevents 6-hydroxydopamine-induced degeneration of neurons in the substantia nigra in vivo. *Proc Natl Acad Sci USA* 96:4078-4083, 1999.
3. Fink D, DeLuca N, Yamada M, Wolfe D, Glorioso JC. Design and application of HSV vectors for neuroprotection. *Gene Ther* 7: (in press), 2000.

V. Presentations

1. Medical University of South Carolina, "Gene Therapy for Neurodegenerative Disease", Charleston, SC – August 1998
2. 5th International Congress of the International Society of Neuroimmunology, Montreal, Canada – August 1998
3. FORGEN-Symposium, "Herpes Virus Gene Vectors for Treatment of Neurologic Disease", Staffelstein, Germany – September 1998
4. 2nd Cellular & Molecular Treatments of Neurological Disease Symposium, "Engineering Virus Vectors for CNS Application", Harvard, MA – October 1998
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7. University of Mexico City, "Application of Herpes Simplex Virus to Cancer Therapy", October 1998
8. Telethon Convention, "Applications of Herpesvirus Vectors to Gene Therapy", Rome, Italy – November 1998
9. Universita Degli Studi Di Ferrara, " ", Milan, Italy – November 1998
10. 1st International Meeting of Gene Therapy of Arthritis & Related Disorders, " " Bethesda, MD – December 1998

11. Keystone Symposium, "Novel Applications Using Herpes Vectors", Salt Lake City, UT – January 1999
12. Cold Spring Harbor (Banbury) Symposium, "Novel Uses of Herpes Vectors", Cold Spring Harbor, NY – May 1999
13. University of Cincinnati, "Altering the Structure and Function with Herpes Virus Vectors", Cincinnati, OH – May 1999
14. Annual Meeting of the Families of Spinal Muscular Atrophy, Scientific Session, "Prospects for Gene Delivery to Motor Neurons using HSV-based Vectors", Milwaukee, WI – June 1999
15. University of Kansas, "Gene Transfer to the Nervous System using Herpes Virus Vectors", Kansas City, KS – June 1999

VI. Conclusion

The results obtained to date demonstrate that an HSV-based gene transfer vector expressing bcl-2 can protect dopaminergic neurons of the substantia nigra from apoptotic cell death induced by 6-OHDA. The construction of a vector expressing GDNF, and the experiments with the latency-active promoter elements will allow us to now determine whether the addition of GDNF provides a synergistic protective effect, and whether vectors employing the latency active promoter in vivo will be able to provide a prolonged biological effect.

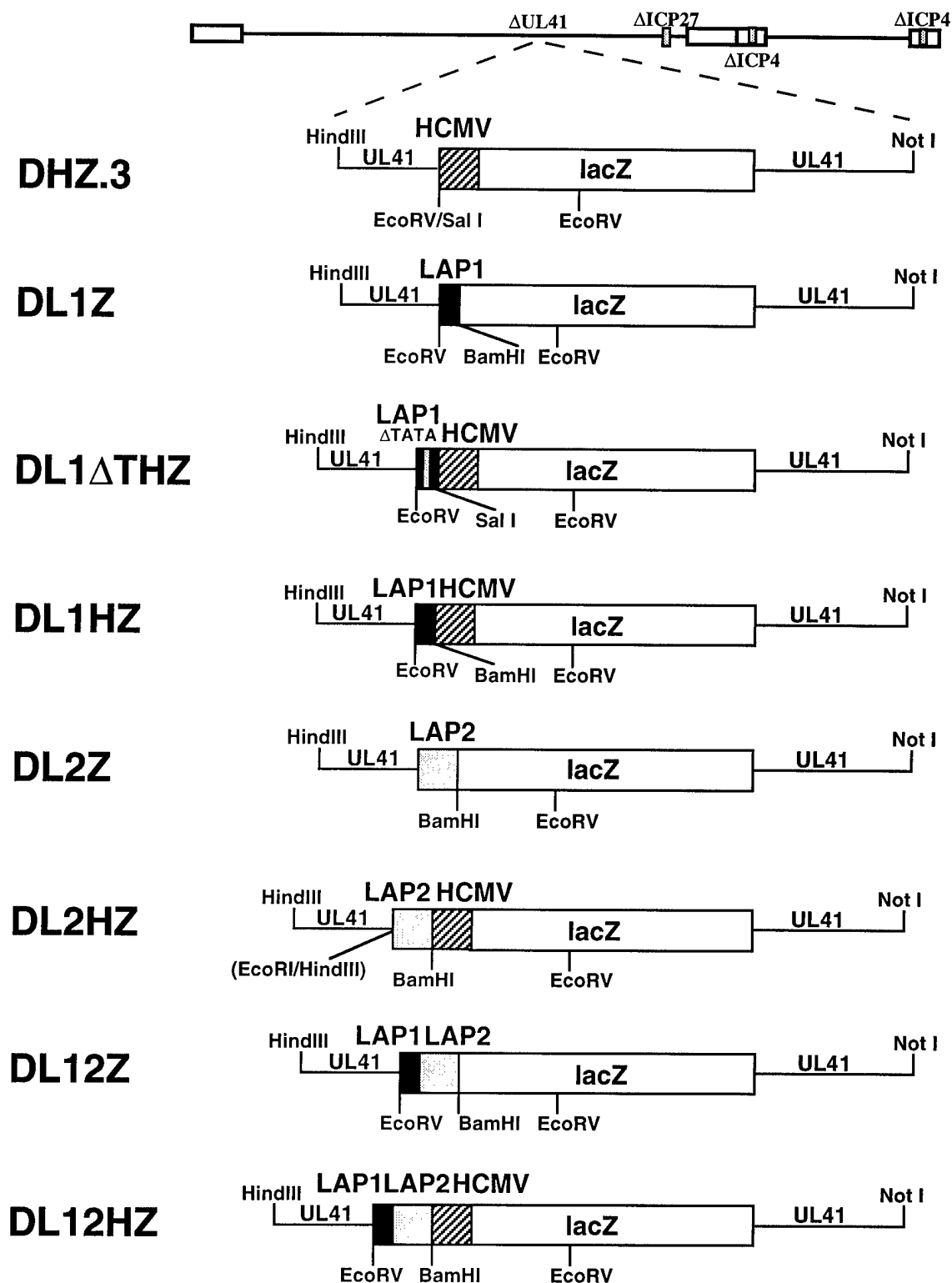


Figure 1. Construction of LAP-HCMV Promoter Expression Recombinant Vectors. A series of lacZ expression constructs were engineered with UL41 flanking sequences and recombined into the UL41 locus of an ICP4-, ICP27- replication defective virus vector. The expression cassettes consisted of either the HCMV IE promoter (DHZ.3), the HSV-1 latency promoter LAP1 (DL1Z), the LAP2 latency promoter (DL2Z), the HSV-1 latency promoters LAP1 + LAP2 (DL12Z), or chimeras containing LAP1-HCMV (DL1HZ), LAP2-HCMV (DL2HZ) or LAP1+2-HCMV (DL12HZ) linked to the lacZ reporter transgene. In addition, a LAP1 promoter with the TATA box deleted was also juxtaposed to HCMV-lacZ (DL1 Δ THZ) to assess the role of the LAP1 TATA in driving transgene expression from the vector. The HCMV IE promoter consists of a 763 bp Sau3A fragment (Thomsen et al., 1984); LAP1 consists of a 203 bp PstI fragment (Devi-Rao et al., 1991; Chen et al., 1995); and LAP2 consists of a 640 bp PstI-BamHI fragment (Goins et al., 1994; Chen et al., 1995).

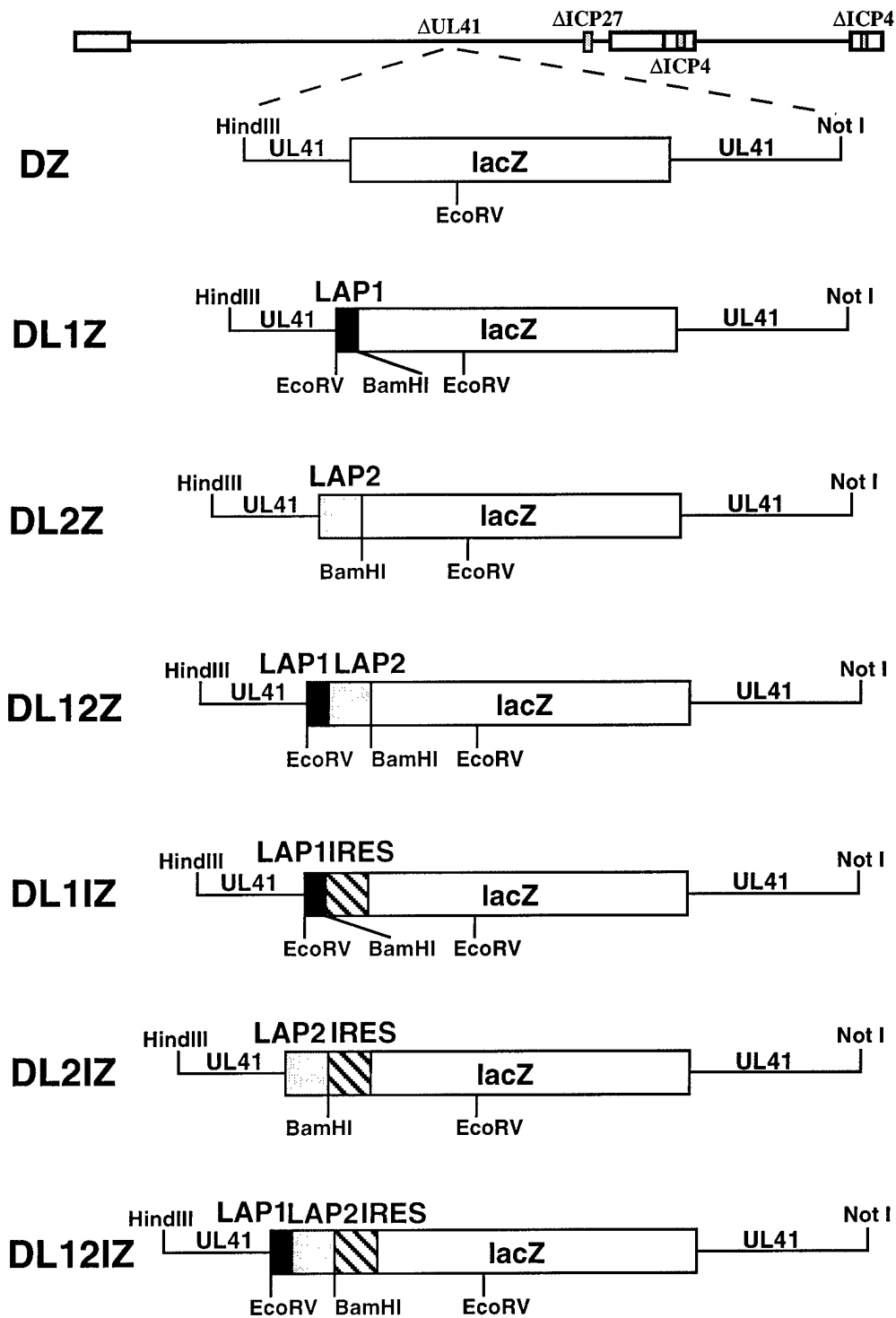
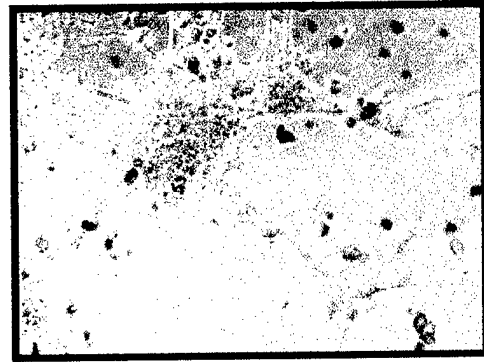


Figure 2. Construction of Replication Defective LAP-IRES Expression Vectors. A series of *lacZ* expression constructs were engineered with UL41 flanking sequences and recombined into the UL41 locus of an ICP4-, ICP27-replication defective virus vector. The expression cassettes consisted of either the HSV-1 latency promoter LAP1 (DL1Z), the LAP2 latency promoter (DL2Z), the HSV-1 latency promoters LAP1 + LAP2 (DL12Z), or constructs in which an IRES was inserted downstream of LAP1 (DL1IZ), LAP2 (DL2IZ) or LAP1+2 (DL12IZ) linked to the *lacZ* reporter transgene. LAP1 consists of a 203 bp PstI fragment (Devi-Rao et al., 1991; Chen et al., 1995); and LAP2 consists of a 640 bp PstI-BamHI fragment (Goins et al., 1994; Chen et al., 1995); and LAP1 + LAP2 consists of the 203 bp PstI fragment of LAP1 linked to the 640 bp PstI-BamHI LAP2 fragment.

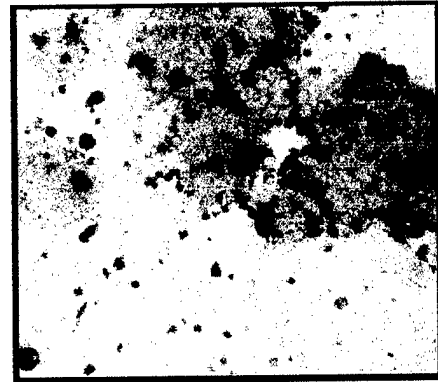
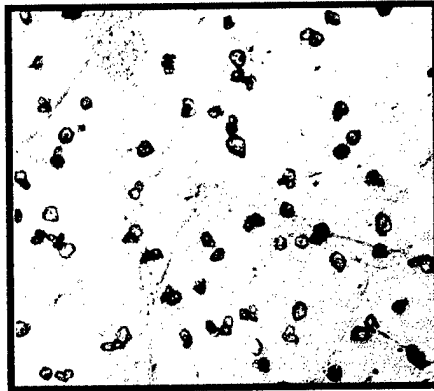
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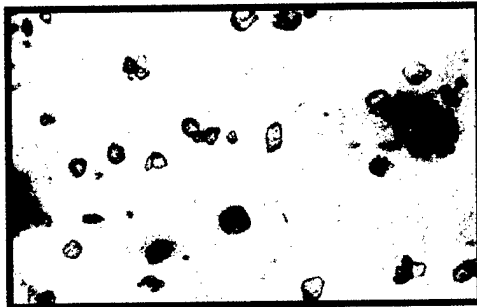
(A) DHZ.3



(B) DL2Z



(C) DL2HZ



(D) DL12HZ

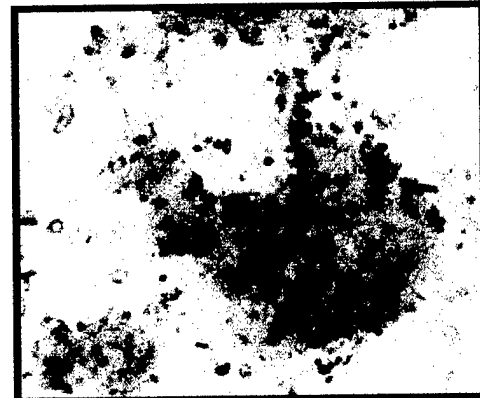


Figure 3. Extended High Level Transgene Expression from Replication Defective Vectors Using LAP-HCMV IE Promoter Chimeric Constructs in Cultured DRG Neuronal Cultures. Primary neuronal cultures from E16 day rat embryos were used to examine whether chimeric LAP-HCMV IE promoter hybrids would be able to drive persistent high levels of transgene from the replication defective vector backbone (ICP4⁻, ICP27⁻). (A) The HCMV promoter alone was active at 2d but waned at 7d (DHZ.3). (B) LAP2 was not active at 2d, but became active by 7d (DL2Z). However, when LAP2 was juxtaposed upstream of the HCMV IEp [(C) DL2HZ or (D) DL12HZ], high levels of transgene were detected at 7d. These constructs continued to express high levels of the transgene even at 28d.

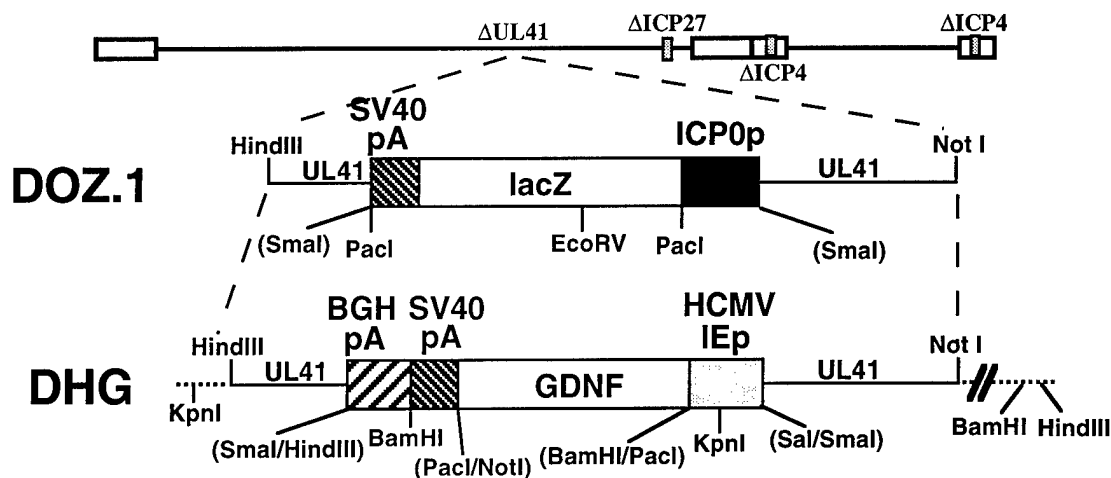
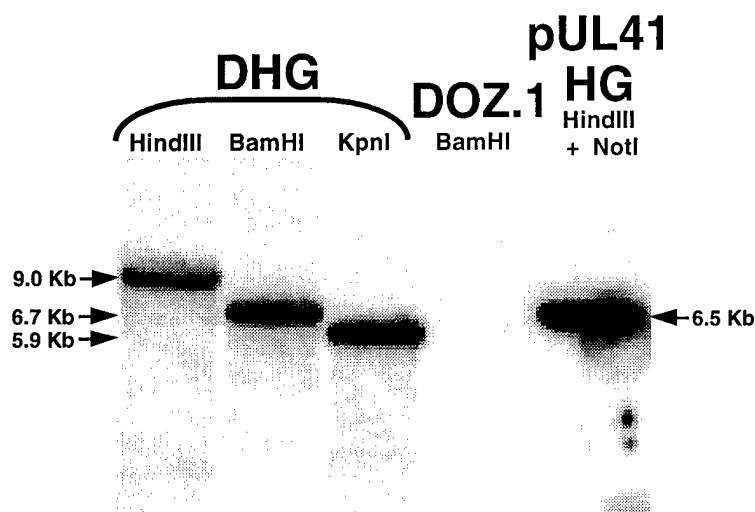
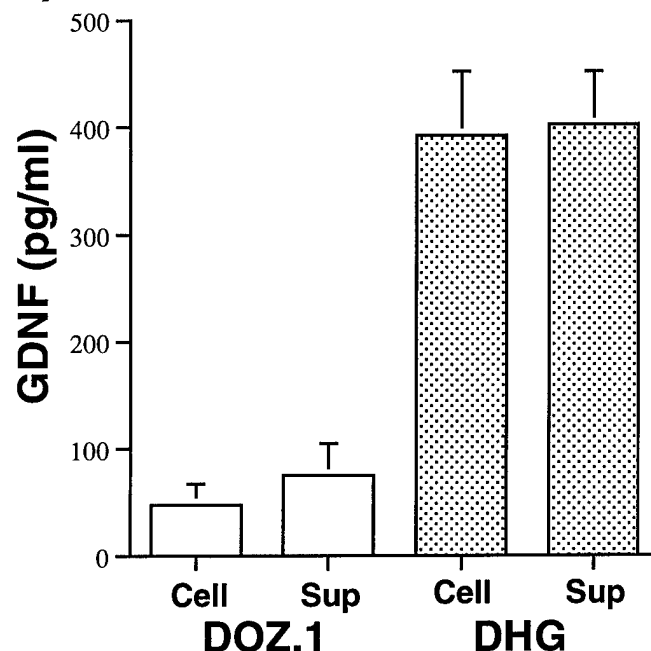
(A)**(B)****(C)**

Figure 4. Construction of Replication Defective GDNF Expression Vectors. (A) An HCMV IEp-GDNF expression construct was engineered with UL41 flanking sequences and recombined into the UL41 locus of an ICP4⁻, ICP27⁻ replication defective virus vector using PacI digestion of the DOZ.1 backbone virus. Recombinants were selected by three rounds of the limiting dilution with the isolation of clear plaques. (B) The genotype of the GDNF recombinant (DHG) was determined by Southern blot analysis using a NotI-BamHI GDNF probe. A positive signal was detected in each of the digestions of the DHG recombinant and the pUL41HG plasmid control, but not in a digest of the DOZ.1. (C) ELISA analysis of Vero cells infected with the GDNF expressing recombinant DHG. Vero cells were infected with the DHG vector and the DOZ.1 control vector (MOI=5) and both cell lysates and supernates were harvested 16 hpi. High levels of GDNF were detected in both the cell pellet and supernatant from DHG-infected cells.

V. Presentations

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8. Telethon Convention, "Applications of Herpesvirus Vectors to Gene Therapy", Rome, Italy - November 1998
9. Università Degli Studi Di Ferrara, "Progress and Development in Application of HSV Gene Vectors", Milan, Italy – November 1998
10. 1st International Meeting of Gene Therapy of Arthritis & Related Disorders, "Herpes Vectors for Gene Therapy", Bethesda, MD - December 1998
11. Keystone Symposium, "Novel Applications Using Herpes Vectors", Salt Lake City, UT – January 1999
12. AFM-DMD Gene Therapy Workshop, "Engineering HSV Vectors for Gene Transfer of the DMD Gene", Paris, France – April, 1999
13. Cold Spring Harbor (Banbury) Symposium, "Novel Uses of Herpes Vectors", Cold Spring Harbor, NY - May 1999
14. University of Cincinnati, "Altering the Structure and Function with Herpes Virus Vectors", Cincinnati, OH – May 1999
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16. University of Kansas, "Gene Transfer to the Nervous System using Herpes Virus Vectors", Kansas City, KS – June 1999
17. American Society of Gene Therapy 2nd Annual Meeting, "Engineering HSV Vectors for Diverse Applications", Washington, DC – June 1999
18. Gene Therapy of Brain Tumor 7th Annual Meeting, "Development of HSV Gene Vectors for Treatment of Glioblastoma", Hiroshima, Japan June 1999
19. Nagoya University School of Medicine, "Engineering HSV Vectors for Diverse Applications", Nagoya, Japan – June 1999
20. National Heart, Lung and Blood Institute Workshop, "Genetically Modified CD34+ Cellular Vehicles for Gene Delivery into Areas of Angiogenesis", Bethesda, MD, July 1999

Engineering Herpes Simplex Virus Vectors for CNS Applications

D. Wolfe,* W. F. Goins,* M. Yamada,* S. Moriuchi,*¹ D. M. Krisky,* T. J. Oligino,*
P. C. Marconi,*² D. J. Fink,† and J. C. Glorioso*

*Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261; and

†Department of Neurology, University of Pittsburgh School of Medicine, and VA Medical Center, Pittsburgh, Pennsylvania 15240

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INTRODUCTION

Several different classes of viruses have been adapted for use as gene transfer vectors, taking advantage of natural viral mechanisms designed to efficiently and effectively deliver DNA to the host cell nucleus. Among these, the human herpesviruses represent excellent candidate vectors for several specific applications. Herpes simplex virus type 1 (HSV-1) is a particularly attractive vehicle for gene transfer to the nervous system since natural infection in humans includes a latent state in which the viral genome persists in a nonintegrated form in neurons, without causing disease in the immune-competent host. However, HSV may be more broadly used for gene transfer applications, since HSV-1 does not require cell division for infection and gene expression, and replication-defective viruses that are effective for gene delivery but do not demonstrate any vector-induced toxicity can be constructed (45, 80). HSV-1 is a large DNA virus with a broad host range that can be engineered to accommodate large or multiple transgene cassettes. Currently available HSV vectors may be generally useful for gene transfer to a variety of tissues in which short-term transgene expression is required to achieve a therapeutic effect. Strategies have been developed to achieve restricted or specifically enhanced vector gene delivery for applications that may require targeting to specific tissues or identified cell types, such as those in which the transgene is cytotoxic (e.g., cancer treatment), or in instances in which improved infection of certain cells (e.g., stem cells) would be highly advantageous.

HSV BIOLOGY

HSV-1 infection is initiated by direct cell contact in a multistep process dependant on several viral glycoproteins (56, 86, 87). Binding of viral glycoproteins gB and

gC to heparan sulfate (HS) (25, 28, 31) is followed by binding of gD to specific cellular receptors to initiate the fusion of cellular and viral membranes (58, 62, 93). Following fusion, the viral capsid is transported to the nuclear membrane where the viral DNA is injected through a nuclear pore, leading to a cycle of lytic viral replication in epithelial cells of skin or mucous membranes. Released viral particles are taken up by nerve terminals and carried by retrograde axonal transport to nerve cell bodies. Latent genomes can persist in sensory neurons for the life of the host, although under the influence of a variety of stimuli wild-type virus may be reactivated from latency, reenter the lytic cycle, and, following anterograde axonal transport to susceptible cells of the dermis, result in recurrent productive virus infection at or near the initial site of infection. As will be described below, recombinant mutant viruses which effectively establish latency but are incapable of lytic replication or reactivation *in vivo* have been constructed.

The HSV genome of 152 kb encodes at least 84 gene products, approximately half of which are nonessential for productive virus infection in some continuous cell lines, but are necessary to efficiently carry out the life cycle of the virus in the host (74). Expression of the viral genes is tightly controlled and occurs in three coordinated waves that have been designated immediate early (IE), early (E), and late (L) (34). IE genes are expressed in the absence of *de novo* viral protein synthesis and their products are responsible for controlling the expression of the other viral functions. E genes encode primarily enzymes and DNA-interactive proteins required for viral DNA synthesis. L genes consist principally of structural proteins of the viral particle. The virus replicates in the nucleus where the capsid is assembled and the newly synthesized viral genomes are encapsidated. The envelope is acquired by budding through the inner lamella of the nuclear membrane (33, 86), after which the particle migrates to the cell surface and is released by exocytosis and cell lysis.

Although wild-type virus can express lytic gene functions in neurons, these infected neurons survive

¹ Present address: Department of Neurosurgery, Osaka University Medical School, 2-2 Yamada-oka, Suita-City, Osaka 565, Japan.

² Present address: Gene Therapy Program, S. Raffaele Hospital, 20132 Milan, Italy.

and, through a set of largely undefined molecular events, lytic gene expression is silenced as the virus enters a latent state. During latency, viral gene expression is limited to an interrelated group of latency-associated transcripts (LATs) (88) which are expressed from the repeat regions flanking the long unique segment (U_L) of the genome and are antisense to and overlap the 3' end of ICP0 mRNA. Two colinear, nonlinear (i.e., common derivative lariat), nonpolyadenylated LAT species of 2.0 and 1.5 kb (12, 15, 91) accumulate in the nuclei of latently infected neurons. These RNAs appear to be stable introns derived from a large, unstable 8.3 kb polyadenylated primary transcript (15, 18, 57, 99). The LAT locus is not absolutely required for the establishment or maintenance of latency, although mutations within the LAT locus may reduce the ability of the virus to establish or reactivate from latency (21, 81–83). The LAT promoter, however, is of great interest since the activity of this promoter might be harnessed to drive transgene expression for prolonged times in appropriate host tissues.

The LAT promoter/regulatory region is composed of two independent latency-active promoters. LAP1 is predominantly responsible for LAT expression during latency (8, 27) while LAP2 is the predominant promoter responsible for LAT expression during lytic infection (8, 27), but is also capable of expressing LAT at low levels in the absence of LAP1 in latently infected animals (R. Ramakrishnan *et al.*, unpublished). LAP1 is located immediately upstream of the region coding for the unstable 8.3 kb LAT and contains many of the basal promoter elements, including the TATA box, ATF/CREB, and USF-1 sites, that have been shown to be required for LAT expression during latency (3, 42, 85), as well as upstream distal elements believed to be involved in neuronal-specific regulation of LAT (92). LAP2, located immediately 5'-proximal to the LAT intron, lacks a TATA box but contains both C/T-rich and poly(T) elements (24, 27) which bind transacting factors that are known to regulate cellular housekeeping gene promoters (24, 43, 70, 78). LAP2 (27), but not LAP1 (50, 53), has been demonstrated to be capable of independently expressing transgenes long term during latency from ectopic locations in defective viral genomes *in vivo*.

REPLICATION-DEFECTIVE-VECTOR DESIGN STRATEGIES

Considerable technical progress has been made in developing HSV vectors through: (i) elimination of vector toxicity, including direct cytopathic effects and the potential inflammatory or immune responses; (ii) full exploitation of the potential transgene capacity of the vector; (iii) maintenance of transgene expression, including the efficiency of cellular transduction, the level and duration of expression, and the potential to

regulate expression; and (iv) targeting of transgene expression to specific cell populations. These advances are described below.

Eliminating Toxicity

The key to eliminating vector toxicity is the elimination of native viral gene expression in the vector. Latent genomes are not toxic because once latency is established lytic cycle gene expression is silenced even in wild-type virus, and only the latency transcripts continue to be produced from the latent genomes, but on initial infection, viral gene expression from the vector genome may be detrimental. Because HSV genes are expressed in a sequential, interdependent order during lytic infection (34), removal of a single essential IE gene (e.g., ICP4) prevents expression of later genes in the gene expression cascade, resulting in a defective vector that is incapable of producing virus particles or expressing E or L gene products, except in complementing cell lines that provide the missing IE gene product (13). However, vectors deleted for only one IE gene continue to express the remaining IE genes (14); with the exception of ICP47, these IE gene products have been shown to be individually toxic to at least some cell types (38) when overexpressed by transfection. The combined elimination of multiple IE genes has been found to reduce the cytotoxicity of HSV-based vectors determined after infection of cell lines or primary neurons *in vitro* (37, 45, 52, 79, 80, 97). For example, a mutant deleted for ICP4, ICP22, and ICP27 transiently arrests DNA synthesis and cell division and expresses a transgene in primary neurons for at least 21 days (45). Mutants deleted for ICP0, ICP4, and ICP27 are less cytotoxic than those deleted for ICP4, ICP22, and ICP27. A mutant deleted for ICP4 and ICP27 has been found to be capable of expressing a lacZ reporter transgene driven by the ICP0 promoter for several days in culture (79). Removal of all IE genes essentially eliminates HSV toxicity for cells *in vitro*, allowing them to be infected at very high multiplicity without any detectable toxicity, similar to UV-irradiated particles that do not express viral functions and are not cytotoxic (80). We have recently constructed an HSV-1 vector deleted for viral functions including the IE genes ICP4, ICP22, and ICP27 to create a mutant background (Fig. 1) for gene therapy applications that require short-term high-level expression of multiple products (44, 45). At multiplicities of infection below 10, this vector background can be used to express transgenes for prolonged times in cultured primary neurons without apparent toxicity (Fig. 1, bottom).

It should be pointed out that toxicity, which can be demonstrated after infection of neuronal cells *in vitro*, is substantially greater than the damage caused by HSV after infection of animals *in vivo*. For example, in the peripheral nervous system (PNS), even wild-type

UL24⁺/
ICP4pTK

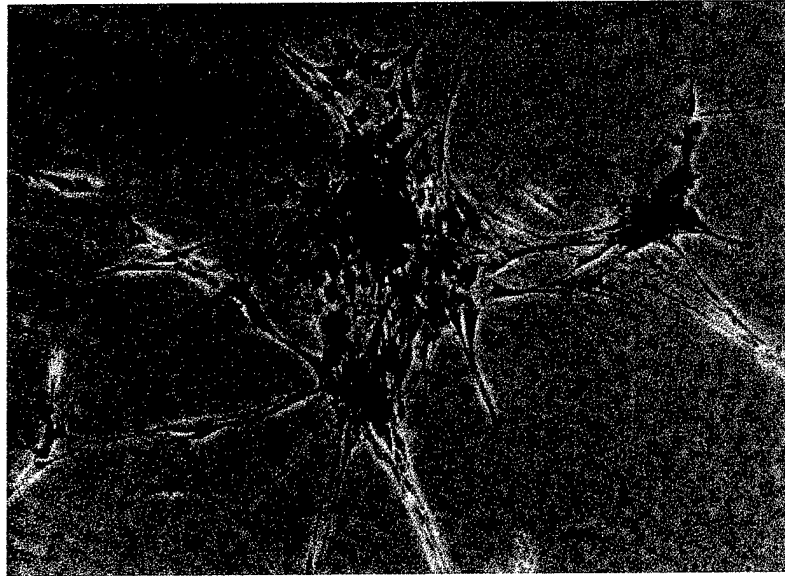
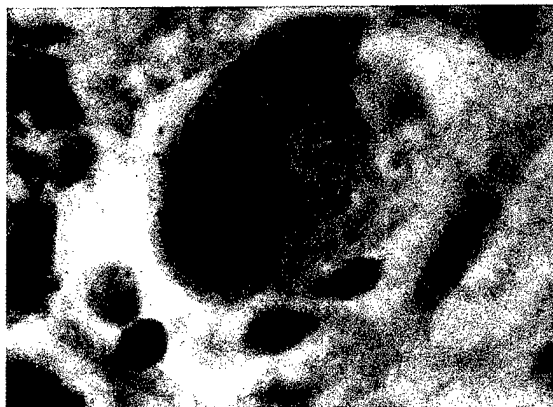
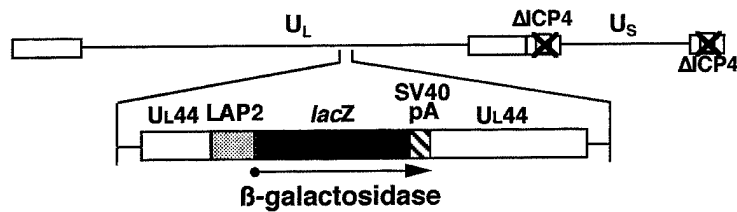
UL41⁺/
ICP0p LacZ

ICP27⁺

ICP4⁺

ICP22⁺

ICP4⁺

**SL2Z**

36

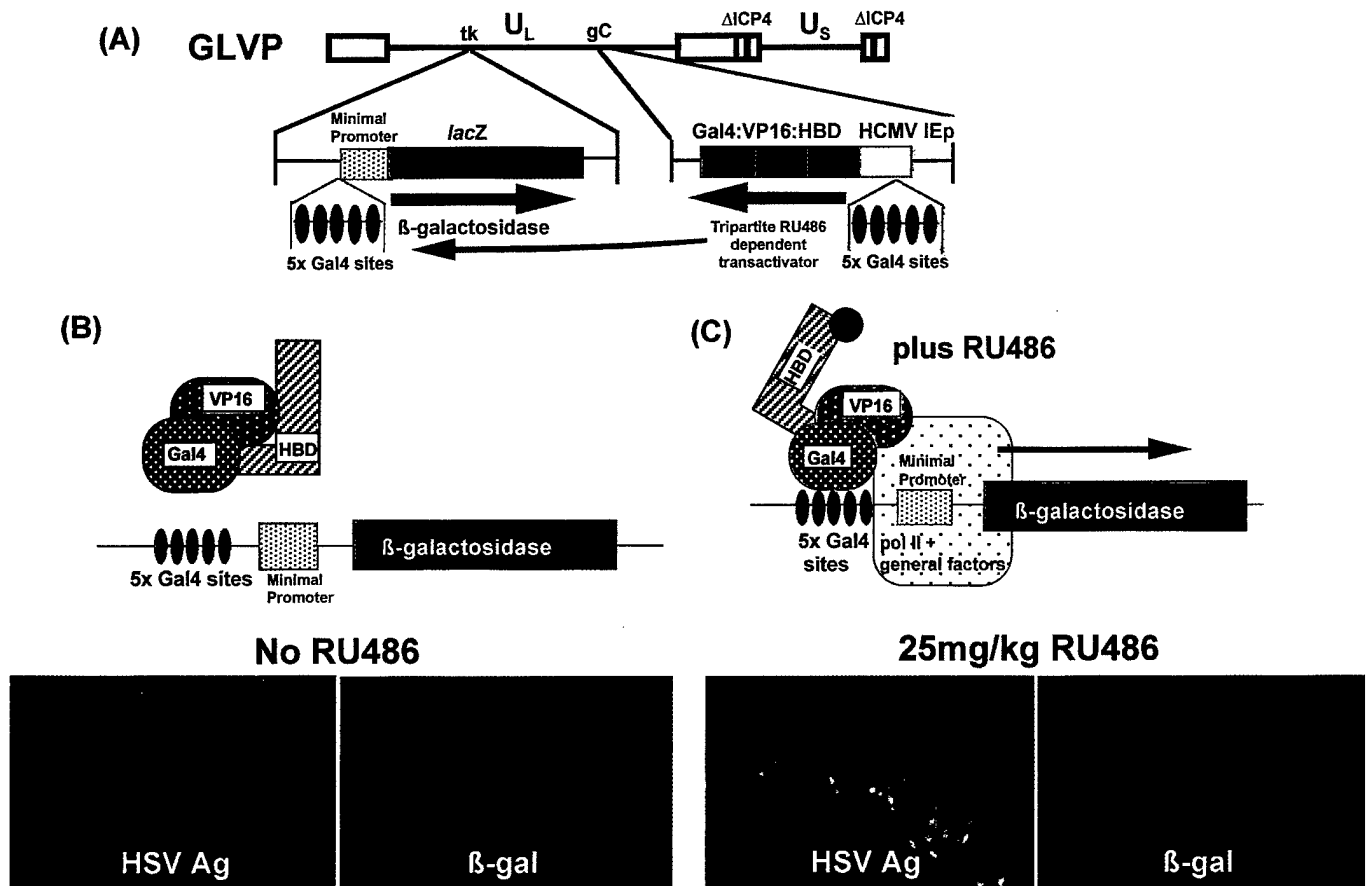


FIG. 4. *In vivo* regulated transgene activation from HSV vectors by a recombinant transactivator. (A) The vector GLVP contains an self-inducible tripartite transactivator (Gal4:VP16:HBD) and *lacZ*, both of which can be activated by RU486. (B) In the absence of the drug, virus can be detected by immunostaining for HSV antigens (HSV Ag), but no β -galactosidase (β -gal) can be seen in the same region. (C) Following administration of RU486 (25 mg/kg) the tripartite transactivator binds to the Gal4 binding sites and abundant β -galactosidase expression is seen by fluorescent immunostaining.

HSV infection of trigeminal ganglion (TG) does not cause histologic damage to the ganglion, and even after recurrent reactivation there is no loss of sensation in the dermatomes innervated by those ganglion, indicating survival of infected TG neurons. In the central nervous system (CNS), we have found that direct stereotactic inoculation of either single (ICP4) or triple (ICP4, ICP22, ICP27) IE-gene-deleted vectors into the substantia nigra is indistinguishable from injection of buffered saline as determined by counts of tyrosine hydroxylase-positive neurons in that brain nucleus at 3 weeks following inoculation (98).

Transgene Capacity

Although early gene therapy applications were designed for the treatment of diseases resulting from single genetic defects, the same methods can be utilized for the delivery of multiple genes for treatment of multigenic or even nongenetic pathologic processes. HSV-1 is ideal for the delivery of multiple genes because of the large size of the HSV genome (152 kb) and

the fact that approximately half of the genome functions may be deleted without blocking viral replication, allowing it to accommodate extensive foreign genetic sequences. We have created a novel HSV-1 vector system in a genetic background suitable for expression of multiple transgenes (44). Together, nine viral genes were deleted, resulting in the removal of 11.6 kb of viral DNA, including the coding sequences for the IE genes ICP4, ICP22, and ICP27 (Fig. 2A). In one group of experiments we inserted into a single vector four independent transgenes at distinct loci under control of different promoters, which include a set of genes with the potential to act synergistically in the local destruction of tumor cells and the induction of anti-tumor immunity. All the transgenes were simultaneously expressed for up to 7 days, with maximum expression occurring at 2 to 3 days postinfection (Fig. 2B). These vectors demonstrate the potential for using HSV-1 vectors for the expression of highly complex sets of transgenes. In the future, it may be advantageous to insert transgenes into immediate early gene loci to

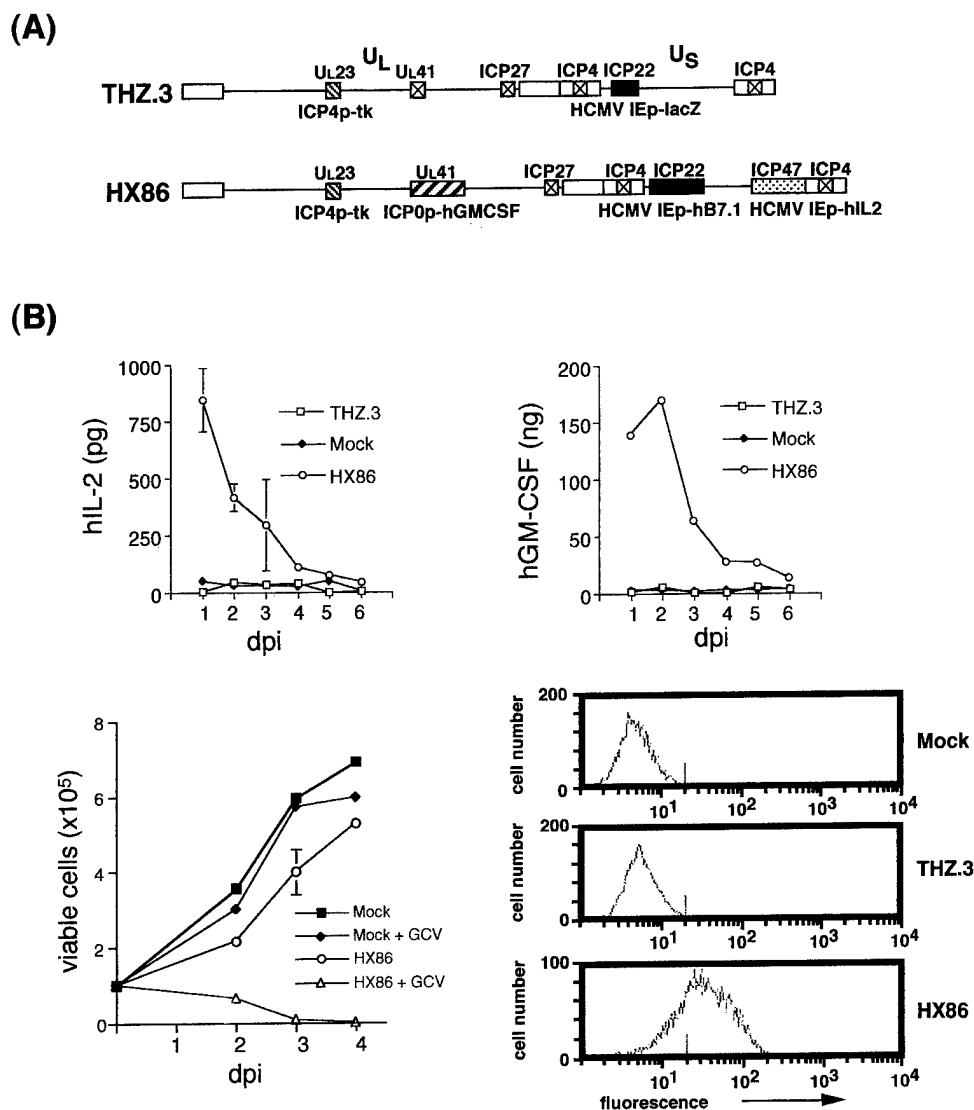


FIG. 2. Multigene vector, transgene expression, and cell viability following infection with control, THZ.3, or multigene, HX86, vector. (A) Schematic diagram of the THZ.3 and HX86 genomes. Deletions are represented as crossed boxes. All other patterned boxes represent the various transgene cassettes. The HSV-1 genome locus is labeled above and the transgene cassettes are labeled below the genome. (B) Transgene expression following infection of 2.5×10^5 Gombos human melanoma cells at a m.o.i. of 5. The expression of the cytokines hIL-2 and hGM-CSF was measured by ELISA. Viable cell counts over time following infection were determined by trypan blue exclusion. Cell surface expression of hB7.1 was determined by FACS analysis 24 h after infection and is displayed as relative fluorescence.

avoid the potential generation of replication-competent virus carrying foreign transgenes through recombination with wild-type virus in latently infected individuals.

Transgene Expression

We have employed lytic HSV and non-HSV gene promoters in the background of replication-defective mutants to drive transgene expression from vector genomes. Both HSV and human cytomegalovirus (HCMV) IE gene promoters produce vigorous transgene expression for up to 1 week after infection (19, 63).

Other viral promoters, such as SV40 (73) and various retroviral LTRs (55), are also highly active for several days in these vector backgrounds. These viral lytic gene promoters are effective for applications that require only transient transgene expression. However, for a number of potential therapeutic applications it will be either necessary or desirable to produce prolonged transgene expression from latent HSV genomes; for this purpose the native latency gene promoters have substantial potential.

Since LAT is the only gene expressed from the viral genome during latency, it should be possible to exploit

the LAT promoter system to express transgenes from persistent genomes in neurons. Both LAP1 and LAP2 have been employed to achieve long-term transgene expression from the HSV vector genome during latency; however, only LAP2 has been capable of long-term expression in neurons. A recombinant with LAP1 driving β -globin expressed β -globin in murine PNS neurons (16), but the level of product dramatically decreased over time (53). Other recombinants with LAP1 driving expression of β -glucuronidase (96), β -galactosidase (50, 53), β -nerve growth factor (NGF) (53), or murine interferon- α (55) either were transiently active or were not active in latently infected animals. Taken together, these results suggest that LAP1 may lack *cis*-acting elements required for long-term expression. However, when LAP1 was juxtaposed either to the Moloney murine leukemia virus LTR (50) or to LAP2 sequences (51) long-term transgene expression was achieved. In contrast to LAP1, LAP2 sequences alone are capable of driving long-term transgene expression when the transgene cassette is introduced into the LAT intron in the native LAT locus (X. Chen *et al.*, unpublished) or when the expression cassette is located in an ectopic locus within the viral genome (26, 27). Reporter transgene expression from these vectors can be detected in neurons of the mouse PNS (Fig. 3) for up to 300 days (27); prolonged expression in the CNS was at low levels, with the *lacZ* RNA detectable only by RT-PCR (X. Chen *et al.*, unpublished).

Because some applications may require enhanced or regulatable transgene expression, we have engineered autoregulatory promoter systems into HSV vectors to control the level or duration of transgene expression. To enhance transgene expression we employed the Gal4/VP16 chimeric transactivator consisting of the HSV VP16 transactivator linked to the yeast Gal4 DNA binding domain, which has previously been shown to be a potent transactivator (76). We created an autoregulatory loop that consisted of a promoter with five tandem copies of the 17-bp yeast Gal4 DNA recognition element juxtaposed to a *lacZ* reporter transgene, to enable transactivation by vector-encoded chimeric Gal4/VP16 protein (Fig. 4A). Completion of the autoregulatory loop by vector-directed Gal4/VP16 expression produced expression of the transgene in transduced neurons in the hippocampus (63). We then modified this system to achieve an inducible promoter system capable of regulatory transgene expression. A transactivator protein consisting of a chimeric molecule made up of the hormone binding domain of the progesterone receptor fused to the transactivation domain of VP16 and DNA binding domain of Gal4 was expressed in an HSV vector under the control of the HCMV IE promoter element (64). The same vector contained the *lacZ* reporter transgene juxtaposed to five tandem copies of the Gal4 DNA recognition element (Fig. 4B). In the

presence of the progesterone analogue RU486, the inactive chimeric transactivator assumes a conformation that allows it to bind to and transactivate the Gal4 recognition site-containing promoter and thus drive transgene expression. Following establishment of vector infection of hippocampus by stereotactic inoculation, we demonstrated that β -galactosidase expression is induced by intraperitoneal injection of RU486 (Fig. 4C).

Targeting

The engineering of HSV vectors to redirect infection to specific cell types will require modification of known viral functions that affect virus attachment, penetration, and spread. Although the envelope structure and mechanism of HSV attachment and entry have been studied in some detail, the complexity of the virus envelope and the fact that multiple glycoproteins are required for virus attachment and entry have made it difficult to assign specific and cooperative functions to individual glycoproteins in these processes.

In order to create an HSV-1 vector for cell type-specific gene delivery, the host range of the vector should be restricted and then redirected. To achieve this goal requires the elimination of the natural tropism of the virus and the subsequent expression of new ligands capable of binding to cell surface receptors characteristic of the targeted cell. Our laboratory has pursued this goal through deletion of the HS-binding capacity of HSV-1, the first step in the process of virus attachment. To eliminate HS binding, we constructed a mutant virus deleted for glycoprotein C (gC) (the major HS-binding protein) and then identified and deleted the HS-binding domain of the essential glycoprotein gB to produce a double-mutant virus which demonstrated an 80% reduction in binding to Vero cells compared to wild-type virus (47). The double-mutant virus displayed a dramatic reduction in binding to the host cell, but penetration was similar to that of wild-type virus, demonstrating that HS binding is not required for virus penetration. This suggests that the HS binding function can be removed and replaced by an alternative ligand thus directing virus binding to a specific ligand receptor.

In our first attempts to redirect binding of the virus, the HS-binding domain of gC was genetically replaced with erythropoietin (EPO) coding sequences to target cells bearing the EPO receptor. The gC:EPO fusion protein was incorporated into budding virions and recombinant gC:EPO virus was specifically retained on a GST:EPO-receptor column. The gC:EPO recombinant displayed a twofold increase in binding to cells expressing the EPO receptor (46). However, this increased binding did not result in productive infection, which normally proceeds via fusion of the viral and cellular membranes, due to endocytosis and subsequent degra-

dation of the receptor-bound virus. Despite the lack of directed infection, this represents the first evidence that HSV-1 tropism can be redirected to a novel non-HSV receptor on the surface of the host cell and that the HS-binding domains of gC can be replaced by novel ligands. These novel ligand:glycoprotein chimeras will enhance binding of virus to specific receptor-bearing cells, although the binding process to these receptors must be redesigned, to target noninternalizing receptors, to effectively result in redirected infection.

APPLICATIONS

Neurodegenerative Disease

One class of diseases that may potentially be treated by HSV-1 vector-mediated gene transfer approaches is the group of conditions broadly known as neurodegenerative diseases, in which continued expression of a therapeutic factor may prevent neuronal death, since the natural biology of the virus enables genomes to persist life-long in the nervous system and the latency promoter system can be employed for long-term therapeutic gene synthesis. Achieving this goal will depend on establishing high-level long-term gene expression in brain and the identification of specific neuroprotective gene products. As described above, we have made considerable progress in the identification of *cis*-elements in the LAPs that may be altered to increase LAP-driven transgene expression in brain. Studies in other laboratories have identified several different products with therapeutic potential in the treatment of neurodegeneration seen in diseases such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (ALS). For example, delivery of glial-derived neurotrophic factor (GDNF) has been shown to prevent the death of nigral dopaminergic neurons induced by 6-hydroxydopamine (6-OHDA) or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in rodent models of Parkinson's disease (9, 89). Delivery of another neurotrophin, NGF, can block the loss of cholinergic phenotype of septal cholinergic neurons following axotomy in rats (94). Administration of the ciliary neurotrophic factor or GDNF can prevent axotomy-induced death of motor neurons and the chronic neurodegenerative characteristic of ALS in the wobbler mouse (36, 49, 77). Delivery of neurotrophins by either systemic administration or intraventricular injection to specific brain regions for treatment of neurodegenerative disease is limited by the blood-brain and brain-CSF barriers. Additionally, many of the neurotrophins possess unwanted cytokine-like side effects which are undesirable and in the case of clinical trials for the treatment of ALS resulted in the use of lower doses of product which lacked beneficial effect (6, 54), unlike what was previously seen in animal models. Limitations, such as limited bioavailability and systemic side

effects, could potentially be overcome through the use of HSV vectors for the specific delivery of neurotrophins.

To address these issues, we engineered a series of HSV vectors that express NGF from either the human HCMV immediate-early promoter or the HSV LAP2 promoter. In mouse TGs *in vivo*, LAP2 produced prolonged NGF expression from the vector backbone. HSV vector-mediated NGF was demonstrated to be neuroprotective for primary dorsal root ganglion neurons in culture (26). We are currently examining the use of these vectors for the treatment of PNS neurodegeneration seen in diabetic neuropathy and diabetic cystopathy in which LAP2-driven, continuous long-term gene expression may prove therapeutic. Similar vectors employing the HCMV IE or HSV latency promoters to express preproenkephalin have been used to regulate the transmission of nociceptive signals at the level of the spinal cord (39, 95) and have potential for the treatment of chronic pain.

In other therapeutic approaches it might be necessary to express a therapeutic product directly in an individual cell in order to ameliorate the disease process. Antisense or ribozyme strategies could be employed to eliminate a toxic product such as the Alzheimer's precursor protein, whose aberrant expression results in neuronal cell death overtime. Similarly, neuronal apoptosis could be blocked through the expression of molecules with antiapoptotic activity, such as Bcl-2. We have demonstrated that replication-defective HSV vectors expressing Bcl-2 protect cortical neurons in culture and neurons in the substantia nigra from 6-OHDA-induced apoptosis (Fig. 5) in a rat model of Parkinson's disease (98).

Cancer

Gene therapy applications for the treatment of cancer rely on methodologies that result in tumor cell death through expression of gene products that either kill tumor cells directly or stimulate anti-cancer immunity. Cancer cell death by direct killing usually involves expression of a transgene that converts a pro-drug such as gancyclovir to a compound toxic for dividing cells. Vaccination strategies include vector-mediated transgene expression that activates or stimulates immunity to the tumor or provides tumor antigens capable of being recognized by the host. Due to the complementary nature of these two approaches, it should be possible to use a combined strategy to treat many forms of cancer since expression of suicide genes (22), cytokines (20), costimulatory molecules like B7 (41), and tumor antigens (30, 67) has been shown to contribute to the recruitment and activation of nonspecific inflammatory responses (32) or the induction of MHC class I or II (7, 84) tumor-specific immunity. Therefore, vectors have been designed to induce specific immunity either

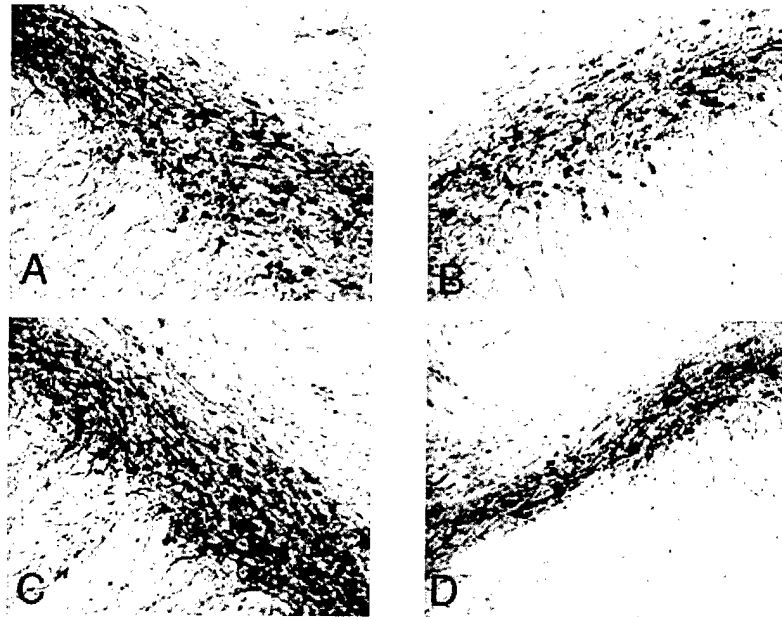


FIG. 5. HSV vector-mediated Bcl-2 expression protects substantia nigral neurons in 6-OHDA unilaterally lesioned animals *in vivo*. Photomicrographs of intact and lesioned vector-injected substantia nigra 2 weeks after 6-OHDA lesion. Representative photomicrographs of TH-immunoreactive cells in intact striatum (A and C) and from an animal injected with THZ/S-bcl2 (B) and TSZ.1 (D). The Bcl2 expression vector increased the number of viable substantia nigra neurons by twofold compared with control virus.

by immunization against a specific tumor antigen displayed by the vector-transduced cell to result in tumor cell death or by introduction of suicide genes into the tumor by direct injection.

The HSV thymidine kinase (tk) or the cellular cytosine deaminase (CD) represent the most commonly used suicide genes employed both in experimental and in human clinical protocols (1, 5, 11, 17, 35, 59, 60, 71). Vector-mediated expression of tk or CD in tumor cells results in tumor cell death following the administration of the antiviral drug gancyclovir (GCV) or 5-fluorocytosine (5-FC), respectively. These drugs, when activated, lead to chain termination or strand-specific breaks during host cell DNA synthesis. An important feature of either the tk-GCV or the CD-5-FC approach is that the transduction of even a low percentage of the tumor cell mass with either of these suicide genes results in significant anti-tumor activity known as the "bystander effect." The bystander effect is the result of cell-to-cell transfer of activated pro-drug (phosphorylated GCV or 5-FC) through gap junctions between vector-transduced tumor cells and neighboring untransduced cells (2, 23, 35, 40).

We have engineered vectors which express high levels of HSV tk under control of an HSV IE gene promoter from the background of a multiply IE-gene-deleted virus (Fig. 6A) to treat human glioblastoma tumors. When these vectors were injected into rodent brain tumors we observed significant increases in survival time, however; tumor progression ensued in all animals, ultimately resulting in death (D. Krisky, P. Marconi, J. Glorioso, unpublished). Thus, a combina-

tion approach may be required in order for suicide gene therapy may to be therapeutic for treatment of tumors.

In order to augment tk-mediated suicide gene therapy, we have combined tk-GCV-mediated tumor cell killing with $\text{TNF}\alpha$ -mediated killing or we have increased the bystander effect of tk-GCV through expression of the gap junction protein Connexin43, which is lowered in many tumor cell types (10, 90). In the first approach, we constructed a vector that expresses $\text{TNF}\alpha$, which has been shown to possess an array of anti-tumor activities including potent cytotoxicity (4, 29), enhancement of the expression of HLA antigens (68) and intracellular adhesion molecule (75) on tumor cell surfaces, and enhancement of interleukin-2 receptors (69) as well as the activation of natural killer cells, lymphokine-activated killer cells, and cytotoxic T lymphocytes (65, 66, 72). Despite the various potential effects of $\text{TNF}\alpha$ as an anti-tumor approach, its clinical use has been limited due to toxic side effects associated with systemic $\text{TNF}\alpha$ delivery (66, 72). In order to direct $\text{TNF}\alpha$ expression specifically to the site of tumor growth and thus minimize systemic side effects, we engineered vectors that express both $\text{TNF}\alpha$ and HSV-tk (Fig. 6B). Both *in vitro* experiments in cell culture and *in vivo* experiments in rodents demonstrated that the effectiveness of HSV-tk suicide gene therapy was enhanced by coexpression of $\text{TNF}\alpha$ (61). In the second approach, we engineered a replication-defective HSV vector expressing both the gap junction protein Connexin43 and HSV-tk (Fig. 6C). As with $\text{TNF}\alpha$, coexpression of Connexin43 and HSV-tk both *in vitro* and *in vivo* increased

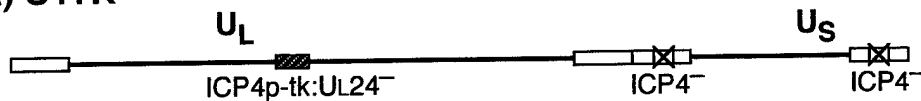
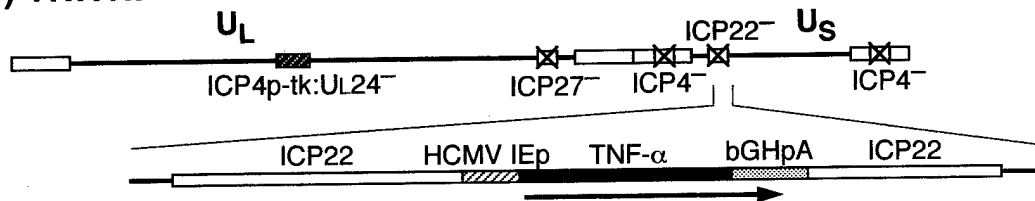
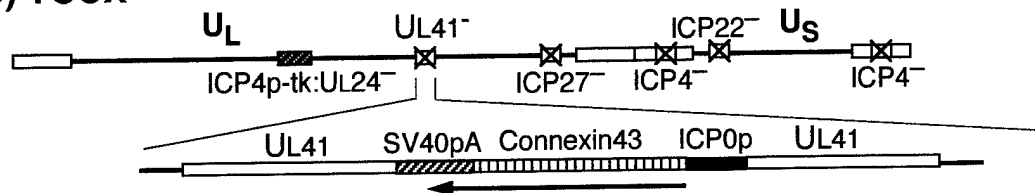
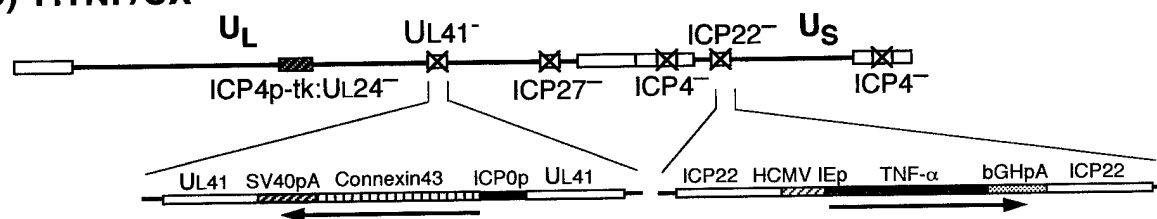
(A) S4TK**(B) TH:TNF****(C) TOCX****(D) T:TNF/CX**

FIG. 6. Replication-defective HSV-1 expression vectors for cancer gene therapy applications. (A) Schematic representation of the S4TK vector (ICP4⁻, U_L24⁻:ICP4p-tk) displaying the HSV tk gene under control of the ICP4 IE gene promoter in the native tk locus. (B) Schematic representation of the TH:TNF vector (ICP4⁻, ICP22⁻:HCMV IEp-TNF-α, ICP27⁻, U_L24⁻:ICP4p-tk) displaying the TNF-α cDNA inserted into the ICP22 locus under the control of the HCMV promoter using the bovine growth hormone (bGH) polyadenylation region. (C) Schematic representation of the connexin 43 vector TOCX (ICP4⁻, ICP22⁻, ICP27⁻, U_L41⁻:ICP0p-Connexin43, U_L24⁻:ICP4p-tk) displaying the Connexin43 cDNA inserted into the U_L41 locus under the control of the ICP0 promoter using the SV40 polyadenylation signal. (D) Schematic diagram of the T:TNF/CX recombinant vector (ICP4⁻, ICP22⁻:HCMV IEp-TNF-α, ICP27⁻, U_L41⁻:ICP0p-Connexin43, U_L24⁻:ICP4p-tk), which expresses TNF-α, Connexin43, and HSV tk.

the GCV-mediated bystander effect in tumor cells in which Connexin43 is expressed at low levels (Marconi *et al.*, unpublished). We have recently isolated a recombinant vector that expresses all three genes (Fig. 6D) and have observed a further increase in the bystander effect in tumor cells *in vitro* (Moriuchi *et al.*, unpublished). Presumably, HSV vector-mediated TNFα and Connexin43 coexpression will augment GCV-activated killing of tumor cells in animal models of brain tumors.

SUMMARY AND FUTURE DIRECTIONS

We have engineered highly defective HSV genomic vectors (i) deleted for multiple IE gene functions which

fail to initiate lytic viral replication, (ii) that express few viral functions, (iii) that display reduced toxicity even for primary neurons in culture (45, 52), (iv) that are able to incorporate multiple transgenes or single large genes (44), (v) that are able to efficiently establish latency in neurons and serve as a platform for long-term gene expression using the latency promoter system, (vi) that cannot reactivate from latency or spread to other nerves of tissues, (vii) that can enhance or regulate transgene expression (63, 64), and (viii) that can be targeted to specific cell types using their normal receptor-recognition ligands modified to contain novel attachment functions (47, 48). These vectors may prove useful in applications in which short-term gene expres-

sion and multiple gene products are required to achieve a therapeutic outcome such as tumor-cell killing (61) and vaccination. Expression of these therapeutic genes may be coordinately regulated (63) or controlled by drug-responsive transactivators (64) that will enable regulation of the level and duration of therapeutic gene expression. Applications that require long-term expression of transgene in PNS neurons, such as diabetic neuropathy, may employ the LAP system for expression of therapeutic genes, such as NGF. Although we have been able to redirect virus binding using novel ligands introduced into the viral glycoproteins, significant improvements will be required to more efficiently bind the virus to the target cell without perturbing normal cellular pathways and still enable the virus to enter the target cell by a mechanism similar to that naturally employed. Future experiments will be designed using vectors that are capable of regulation of therapeutic gene expression using their own native promoters, thus responding to the natural stimuli involved in their production. Further work will be necessary to modify the vector for non-nervous-system approaches to treat diseases of other tissues such as arthritis, muscular dystrophy, or autoimmune disorders.

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Herpes simplex virus vector-mediated expression of Bcl-2 prevents 6-hydroxydopamine-induced degeneration of neurons in the substantia nigra *in vivo*

MASANOBU YAMADA*†, THOMAS OLIGINO†‡, MARINA MATA*§, JAMES R. GOSS*§, JOSEPH C. GLORIOSO‡, AND DAVID J. FINK*‡§

Departments of *Neurology and ‡Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213; and §Veterans Affairs Medical Center, Pittsburgh, PA 15240

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ABSTRACT 6-Hydroxydopamine (6-OHDA) is widely used to selectively lesion dopaminergic neurons of the substantia nigra (SN) in the creation of animal models of Parkinson's disease. *In vitro*, the death of PC-12 cells caused by exposure to 6-OHDA occurs with characteristics consistent with an apoptotic mechanism of cell death. To test the hypothesis that apoptotic pathways are involved in the death of dopaminergic neurons of the SN caused by 6-OHDA, we created a replication-defective genomic herpes simplex virus-based vector containing the coding sequence for the antiapoptotic peptide Bcl-2 under the transcriptional control of the simian cytomegalovirus immediate early promoter. Transfection of primary cortical neurons in culture with the Bcl-2-producing vector protected those cells from naturally occurring cell death over 3 weeks. Injection of the Bcl-2-expressing vector into SN of rats 1 week before injection of 6-OHDA into the ipsilateral striatum increased the survival of neurons in the SN, detected either by retrograde labeling of those cells with fluorogold or by tyrosine hydroxylase immunocytochemistry, by 50%. These results, demonstrating that death of nigral neurons induced by 6-OHDA lesioning may be blocked by the expression of Bcl-2, are consistent with the notion that cell death in this model system is at least in part apoptotic in nature and suggest that a Bcl-2-expressing vector may have therapeutic potential in the treatment of Parkinson's disease.

6-Hydroxydopamine (6-OHDA) is a neurotoxin taken up into dopaminergic neurons through the dopamine transporter, where the compound is autooxidized to form semiquinone and superoxide anions that subsequently are converted to hydroxyl radicals through interaction with H₂O₂ (1, 2). Injection of the toxin into striatonigral projections results in selective damage to dopaminergic neurons and has been used to create widely used models of Parkinson's disease in rats (3, 4). Neuronal injury after direct injection of the toxin into the region of the substantia nigra is acute (5), but a subacute lesion that progresses over weeks can be created by injection of 6-OHDA into the proximity of the dopaminergic terminal in the striatum. Morphologic (6, 7) behavioral and biochemical effects (8, 9) have been demonstrated with this model.

6-OHDA lesioning of dopaminergic terminals in the striatum of neonatal rats *in vivo* results in the appearance of apoptotic cell death in phenotypically defined dopaminergic neurons over the course of 10 days postlesioning (10). In older animals the appearance of dying cells suggests a combination of necrotic and apoptotic cell death (10). PC-12 cells induced to die by exposure to 6-OHDA *in vitro* demonstrate cell shrinkage, chromatin condensation, membrane blebbing, and

DNA fragmentation characteristic of apoptosis (11). Additional evidence that cell death in this model proceeds through apoptotic mechanisms is suggested by the up-regulation of p53 and Bax protein expression after 6-OHDA exposure (12) and the observation that cell death can be blocked by addition of a caspase inhibitor (13). Cell death with characteristics of apoptosis also has been demonstrated after exposure to L-dopamine in PC-12 cells (14) and sympathetic neurons (15), suggesting that endogenous oxidative stressors may produce similar toxicity to these cells.

Bcl-2, the prototypical member of a family of at least 12 related proteins that are structural and functional homologues of the nematode protein CED-9 (16), is capable of protecting cells against apoptotic cell death resulting from a variety of cytotoxic insults (17). Bcl-2 is located primarily on the cytoplasmic face of the mitochondrial outer membrane, endoplasmic reticulum, and nuclear envelope. Bcl-2 blocks activation of the caspase cascade and also protects mitochondria, preventing the opening of permeability transition complexes and the release of cytochrome *c* (18). Dopamine-induced apoptosis of PC-12 cells is inhibited by the overexpression of Bcl-2 (19), as is metamphetamine-induced cell death in an immortalized neuronal cell line (20). Cultured striatal neurons from transgenic mice overexpressing Bcl-2 similarly are resistant to 6-OHDA toxicity *in vitro* (21).

To determine the contribution of apoptotic cell death to the loss of tyrosine hydroxylase (TH)-expressing neurons of the substantia nigra (SN) after 6-OHDA lesioning of the striatum *in vivo*, we constructed a multiply deleted replication-incompetent genomic herpes simplex virus (HSV) vector carrying the coding sequence for Bcl-2 under the transcriptional control of the simian cytomegalovirus immediate early promoter (SCMV IEP). Injection of the vector into the striatum 1 week before lesioning with 6-OHDA resulted in a 50% increase in the number of surviving TH-immunoreactive and retrogradely labeled fluorogold (FG) neurons in the substantia nigra 2 weeks after lesioning.

MATERIALS AND METHODS

Generation of a Bcl-2 Expressing Vector. A multiply deleted replication-incompetent genomic HSV vector expressing the human Bcl-2 gene product was constructed from a recombinant plasmid containing a Bcl-2 expression cassette flanked by sequences from the HSV genome. The human bcl-2 cDNA

This paper was submitted directly (Track II) to the *Proceedings* office. Abbreviations: 6-OHDA, 6-hydroxydopamine; SN, substantia nigra; TH, tyrosine hydroxylase; FG, fluorogold; HSV, herpes simplex virus; RT-PCR, reverse transcription-PCR; TUNEL, terminal deoxynucleotidyltransferase-mediated UTP end labeling; GDNF, glial-derived neurotrophic factor.

†M.Y. and T.O. contributed equally to this work.

§To whom reprint requests should be addressed at: S-520 BST, 200 Lothrop Street, Pittsburgh, PA 15213. e-mail: dfink@med.pitt.edu.

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was obtained from the ATTC clone number 79804 as an *EcoRI*-*NsiI* fragment. The cDNA coding for the 5' untranslated sequence from the rabbit β -globin gene, including the second intron, was added upstream of the translation start site. The cDNA was modified further by the addition of the β -globin 3' untranslated region and simian virus 40 polyadenylation sequences downstream of the stop codon, and this modified cDNA was placed under the control of the SMCV IEp (22) in a plasmid containing sequences from the HSV thymidine kinase (TK) locus. The expression cassette was flanked by HSV sequences 48634–47978 upstream and 47416–46098 downstream. (All HSV genome numbers refer to the published sequence for HSV strain 17.) The cloning of the expression cassette into this locus resulted in a deletion of the tk gene from 47416–47978, which inactivates the gene. Recombination of the plasmid into the virus genome yields recombinants that are tk⁻ and therefore resistant to treatment with ganciclovir. The vector THZ/S-bcl2 (Fig. 1) was created by homologous recombination of the plasmid into the TK locus of the ICP4-deleted HSV vector d120 (23) using standard calcium phosphate precipitation (24). Recombinants were identified by selection of progeny virus with ganciclovir (5 μ g/ml), isolated, and tested for the presence of the bcl-2 gene by Southern blot analysis. Isolates that contained the bcl-2 cassette (d120/S-bcl2) were purified by three rounds of limiting dilution. The d120/S-bcl2 vector was crossed genetically with the ICP4-, ICP22-, and ICP27-deficient vector THZ.1 (25) by infecting complementing 7B cells with both vectors at a multiplicity of infection (moi) of 5, and ganciclovir-resistant plaques that expressed lacZ were selected. The deletion in ICP27 was confirmed by the inability of the isolates to grow on ICP4-complementing cell lines and Southern blot. The final vector was grown to high titer, purified on a Nicodenz gradient, and used in all the experiments described. Two control vectors, similarly deleted for ICP4, ICP22, and ICP27, were used. THZ.1 had the lacZ reporter gene under the control of the HCMV IEp in the ICP22 locus, whereas TSZ.1 was constructed with an SMCV IEp:lacZ reporter cassette in the tk locus of the recombinant HSV vector genome (Fig. 1).

Cell Culture and *in Vitro* Transfections. Hig-82 cells were cultured in MEM at 37°. Neuronal cultures were prepared from cortex from 17-day-old rat embryos, which were dissociated with 0.25% trypsin-1 mM EDTA (30 min at 37°) and

plated on poly-D-lysine (molecular weight, 70,000–150,000; Sigma)-coated coverslips at 2×10^5 cells per well (24-well plate; Falcon) in defined medium [NeuroBasal Medium supplemented with B-27 (1 \times concentration), Glutamax I (0.5 ml/50 ml) and Albumax II (50 μ l/50 ml), all purchased from GIBCO/BRL], and incubated at 37° in humidified 95% air, 5% CO₂. These cultures contain greater than 95% neurons as determined by immunostaining with antibodies directed against neurofilament and glial fibrillary acidic protein (data not shown). Seven days after plating, cultures were infected at an moi of 1 in 250 μ l of medium, for 1 hr at 37°, with gentle agitation every 10 min. Bcl-2 protein was detected by Western analysis. Forty-eight hours after infection cells were lysed in Laemmli buffer, the protein was separated by 12% SDS/PAGE and transferred to nitrocellulose membrane, which was probed with an anti-Bcl-2 antibody (1:100, Boehringer Mannheim) and detected with an alkaline phosphatase-conjugated secondary antibody (1:2,000; Sigma). Similar numbers of cells were harvested for mock, control, and vector-infected samples, and Ponceau-S staining confirmed the transfer of equivalent amounts of protein per lane. The amount of Bcl-2 in the cell lysate was also determined by ELISA by using a commercially available kit (Endogen, Cambridge, MA) following the manufacturer's instructions. The number of viable cells was determined by Alamar blue fluorescence. Alamar blue (2% final concentration; Alamar Biosciences, Westlake, OH) was added to the cultures, and after 4-hr incubation at 37°, the viable cells were determined by quantitative fluorescence (530/590, excitation/emission) as described. For immunohistochemistry, cells were fixed in cold 4% paraformaldehyde, incubated overnight at 4° with the primary antibody (anti-Bcl-2 1:100, Boehringer Mannheim; anti-MAP2 1:1,000, Sigma), followed by a CY3-conjugated secondary antibody (1:3,000, Sigma) for 1 hr at room temperature. Apoptotic nuclei in uninfected cultures were identified by terminal deoxynucleotidyltransferase-mediated UTP end labeling (TUNEL). Uninfected cells were fixed in 4% paraformaldehyde, rinsed once with PBS, and permeabilized with 0.17% Triton X-100/0.1% sodium citrate for 2 min on ice, followed by incubation with the TUNEL reaction mixture of the fluorescein *in situ* cell death detection kit (Boehringer Mannheim) following the manufacturer's instructions. Negative controls, incubated without enzyme, and positive controls, treated with DNase I, produced appropriate results.

Animal Experiments. 6-OHDA lesioning was carried out according to the protocol of Sauer and Oertel (6). Female Sprague-Dawley rats (200–225 g) were injected into the striatum bilaterally (coordinates anterior-posterior +1.0, medial-lateral 3.0, dorsal-ventral -5.0 relative to the bregma) with the retrograde tracer fluorogold (0.2 μ l of 2% FG in 0.9% saline; Fluorochrome, Denver, CO), and at the same time injected unilaterally into the region of the SN (AP -5.3, ML 1.9, DV -7.4) with either vector or control (4 μ l at 1 μ l/min). At 1 week after inoculation, 6-OHDA (16 μ g of free base, 0.2 μ g/ μ l in 0.9% saline) was injected unilaterally into striatum using the same coordinates as the fluorogold, ipsilateral to the vector injection into SN. The experiment was performed with nine animals in each group, and the entire experiment was repeated once with identical results.

Reverse Transcription-PCR (RT-PCR). Transgene expression of bcl-2 RNA was examined by RT-PCR. Total RNA was isolated from one glass slide-mounted 40- μ m section from three vector-injected and two control animals, using TRIZOL Reagent (GIBCO/BRL) as per the manufacturer's instructions. Approximately 1 μ g of RNA was treated with DNase I (Boehringer Mannheim) for 45 min at 42° and then inactivated by incubation at 100° for 5 min. First-strand cDNA was made by using a GeneAmp kit (Perkin-Elmer) as per the manufacturer's instructions and approximately 0.2 μ g of DNase-treated total RNA. Two 40-cycle PCR amplifications, using nested

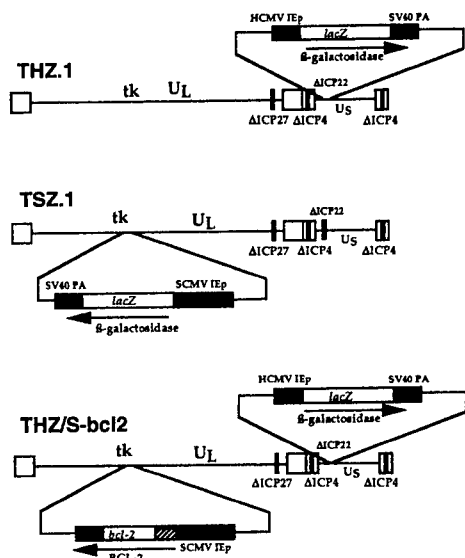


FIG. 1. Schematic representation of genomic HSV vectors. Both the control vectors THZ.1 and TSZ.1 and the Bcl2-expressing vector THZ/S-bcl2 are deleted for the immediate early genes ICP4 (both copies), ICP27, and ICP22, indicated by solid bars in the genome.

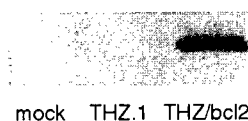


FIG. 2. Bcl-2 expression *in vitro*. Western blot of lysates of infected Hg-82 cells shows a 26-kDa BCL-2 immunoreactive band in THZ/S-bcl2 but not mock- or THZ.1-infected cells.

primers to the human Bcl-2 sequence, were performed by using a Perkin-Elmer GeneAmp 2400 thermocycler and Perkin-Elmer AmpliTaq Gold enzyme (20 μ l total reaction volume, consisting of 0.2 mM dNTP, 10 pM primers, and 0.5 unit of *Taq* polymerase). Four microliters of cDNA reaction product was used as template for the first round of amplification (forward primer, 5'-gaattccactgtcaagaagagcagt-3'; reverse primer, 5'-atgctgtgttgatattcgaaagc-3'; GIBCO/BRL), and 4 μ l of the first amplification product was used as template for the second round of amplification (forward primer, 5'-acagaggccctggccttcctat-3'; reverse primer, 5'-agttccaggtgtg-gaatatggg-3'; GIBCO/BRL). Cycle conditions were 94° for 1 min, 58° for 1 min, and 72° for 2 min.

Histology and Cell Counting. Two weeks after 6-OHDA lesioning the animals were sacrificed by intracardiac perfusion with 4% paraformaldehyde in PBS, the brains were cryoprotected with 30% sucrose, and 40- μ m sections were mounted directly for examination of FG-labeled cells or processed for immunocytochemistry. For TH-immunocytochemistry, float- ing sections were incubated with an anti-TH antibody (1:500,

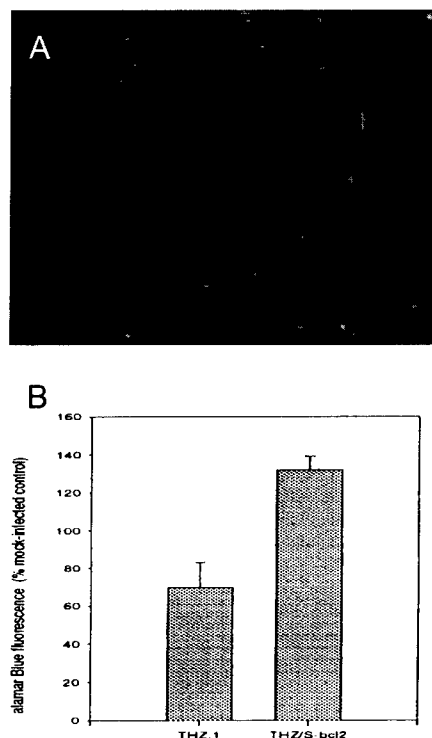


FIG. 3. Expression of Bcl-2 in cortical neurons *in vitro* inhibits naturally occurring cell death. (A) TUNEL staining of uninfected primary cortical neuron cultures at 3 weeks. The majority of residual shrunken nuclei in uninfected cultures was TUNEL-positive, whereas viable neurons were unlabeled. Similar patterns were seen in cultures at 2 and 4 weeks after plating. (B) Three weeks after infection, cultures infected with THZ/S-bcl2 demonstrated increased cell survival compared with uninfected cultures or cultures infected with THZ.1. Cell viability was determined by Alamar blue fluorescence, and the data are presented as a percentage of mock-infected cells. The data presented were obtained from three independent repetitions of the same experiment, with four wells in each group for each experiment. ($P < .05$ for the comparison of THZ.1 with THZ/S-bcl2 by *t* test.)

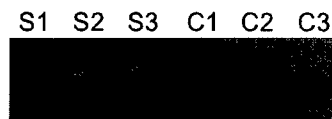


FIG. 4. Transgene-driven expression of human Bcl-2 RNA in rat SN. RT-PCR from a single 40- μ m section of rat SN demonstrates bcl-2 in vector-injected (S1-S3) but not control (C1-C2) brains. C3 is a water blank.

overnight at room temperature; Chemicon), followed by a secondary antibody conjugated to biotin (1:200 for 2 hr; Vector Laboratories), and detected with diaminobenzidine by using a commercial kit (Vector Elite; Vector Laboratories). The number of surviving FG-labeled and TH-immunoreactive neurons was determined by counting labeled or immunoreactive cells through the SN (four to seven sections per animal for each technique), and the total number was expressed as a

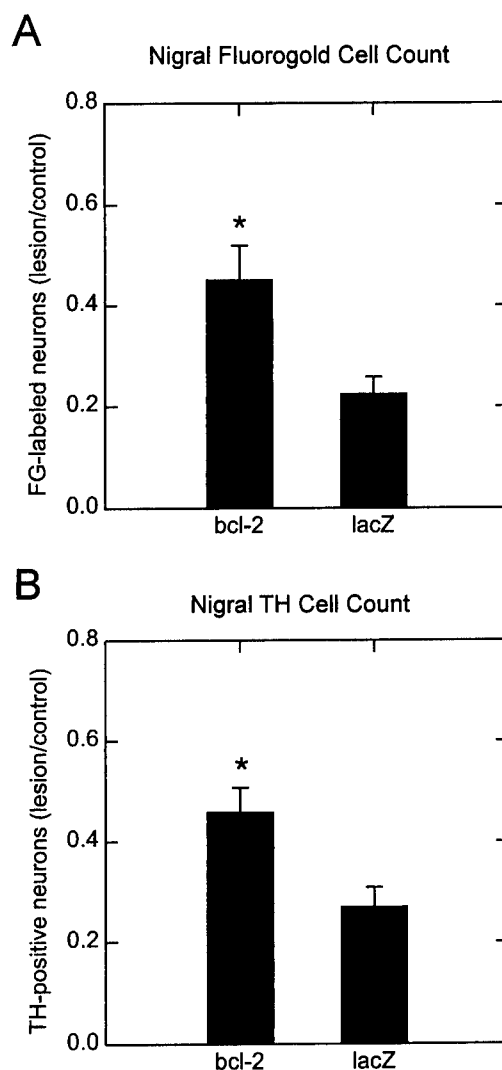


FIG. 5. Expression of Bcl-2 in SN protects neurons from 6-OHDA toxicity. (A) Cell survival as estimated from cell counts of nigral FG⁺ cells 2 weeks after 6-OHDA lesion. THZ/S-bcl2 transfection resulted in a significantly greater number of FG⁺ cells surviving the lesion as compared with THZ.1 control transfected SN ($n = 9$ animals in each group, $P < .05$). (B) Cell survival as estimated from counts of TH-immunoreactive cells 2 weeks after 6-OHDA lesion. THZ/S-bcl2 transfection resulted in a significantly greater number of TH-immunoreactive cells surviving the lesion as compared with THZ.1 control transfected SN ($n = 9$ animals in each group, $P < .05$). The experiment was repeated twice with the same results.

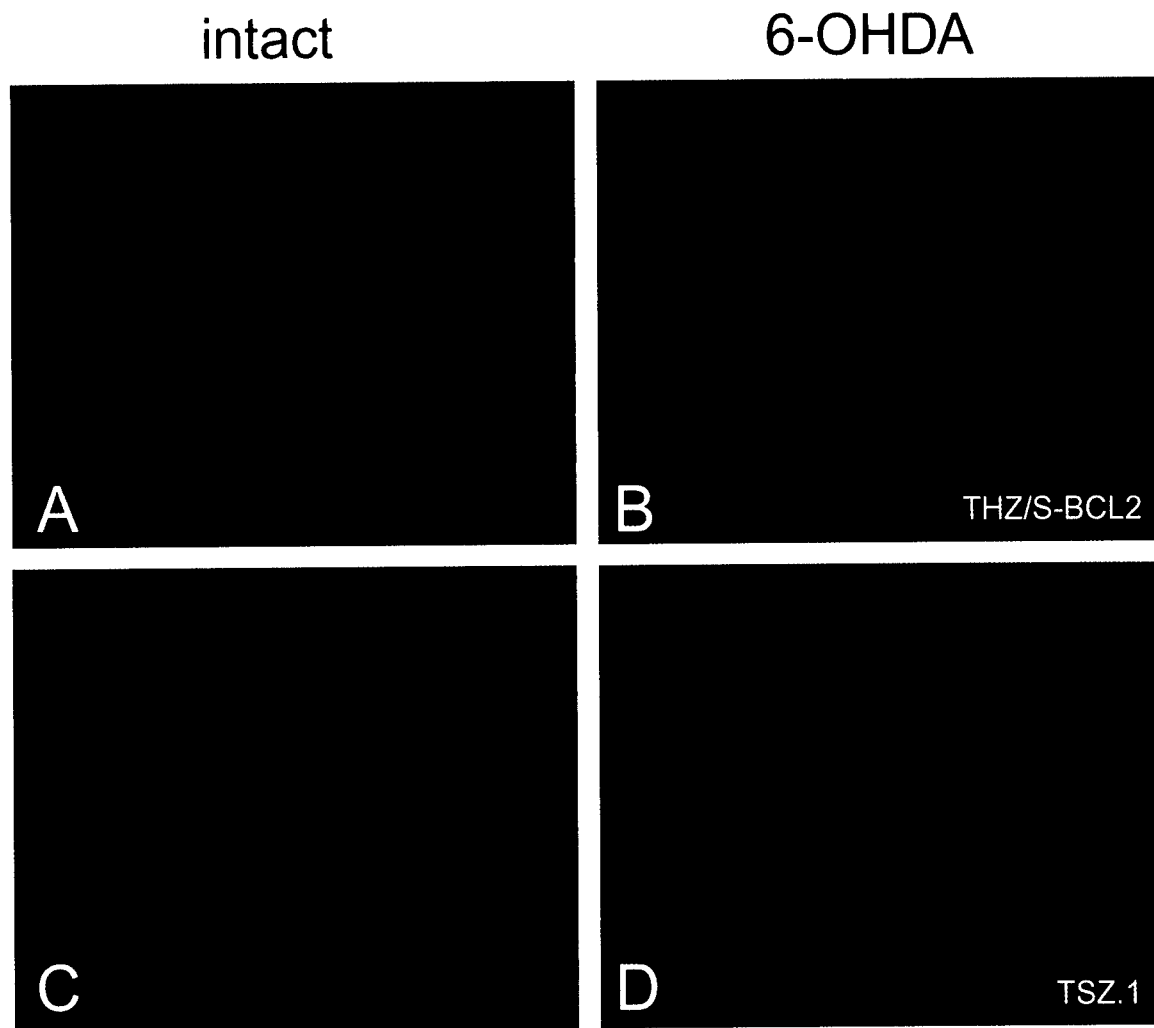


FIG. 6. Photomicrographs of intact and lesioned/vector-injected SN 2 weeks after 6-OHDA lesion (FG⁺ cells). Representative photomicrographs of FG⁺ cells in contralateral intact SN (A and C) and ipsilateral SN injected with THZ/S-bcl2 (B) and TSZ.1 (D) are shown.

percentage of the unlesioned side. The statistical significance of the difference between the two sides was determined by *t* test (Systat, Evanston, IL).

RESULTS

THZ/S-bcl2 Vector Expresses Bcl-2. The ability of the THZ/S-bcl2 vector to express biologically active Bcl-2 first was determined *in vitro*. Hg-82 cells, infected at an moi of 1, were harvested 48 hr postinfection. A 26-kDa bcl-2 immunoreactive band was found in the lysate of THZ/S-bcl2-infected cells, but not in uninfected or control vector infected cells (Fig. 2). The amount of Bcl-2 in the lysate of infected cells was approximately 400 pg/well as determined by ELISA. Control vector (THZ.1) infection produced essentially no detectable Bcl-2 (<25 pg/well). The biological activity of vector-mediated Bcl-2 was assessed after infection of primary cortical neurons in culture. These cells, grown in defined medium in the absence of trophic factors, undergo naturally occurring cell death between 2 and 4 weeks in culture. The apoptotic nature of cell death in this model system was confirmed by TUNEL staining of the remaining nuclei at 3 weeks after plating (Fig. 3A). Cultures were infected with the control vector (THZ.1) or the bcl-2-expressing vector (THZ/S-bcl2), and 3 weeks postinfection the number of viable cells were determined by Alamar blue fluorescence. Cultures infected with the Bcl-2-producing vector had twice as many surviving cells as control virus-infected cultures and 130% of uninfected cultures (Fig. 3B).

The identity of surviving cells as neurons was confirmed by microtubule-associated protein 2 (MAP2) immunoreactivity (data not shown). These cells were larger and had longer and larger processes than the cells in uninfected or control-infected cultures.

THZ/S-bcl2 Vector Protects SN Neurons from 6-OHDA Toxicity. The effect of transgene expression of Bcl-2 *in vivo* was assessed by striatal 6-OHDA lesioning after injection of the vector into the region of the SN. Expression of the human bcl-2 RNA in vector-injected rat SN was confirmed by RT-PCR (Fig. 4). Bcl-2 produced from the THZ/S-bcl2 vector prevented 6-OHDA-induced degeneration of retrogradely labeled FG-positive neurons (Figs. 5A and 6) as well as the degeneration of TH⁺ dopaminergic cells of the SN (Figs. 5B and 7). Approximately twice as many cells of the SN, labeled retrogradely by FG or identified by the TH immunoreactivity, survived 2 weeks postlesioning in the bcl-2 vector-treated animals compared with control vector-injected animals. Injection of the bcl-2 vector or the control vector alone, without 6-OHDA lesioning, resulted in approximately 40% reduction in the number of TH⁺ or FG-labeled cells on the injected side at 3 weeks postinjection, although inspection of hematoxylin/eosin-stained sections revealed no evidence of an inflammatory infiltrate in animals sacrificed 3 weeks after vector injection (data not shown).

DISCUSSION

The data presented in this study demonstrate that expression of bcl-2 in the SN protects dopaminergic neurons of the SN

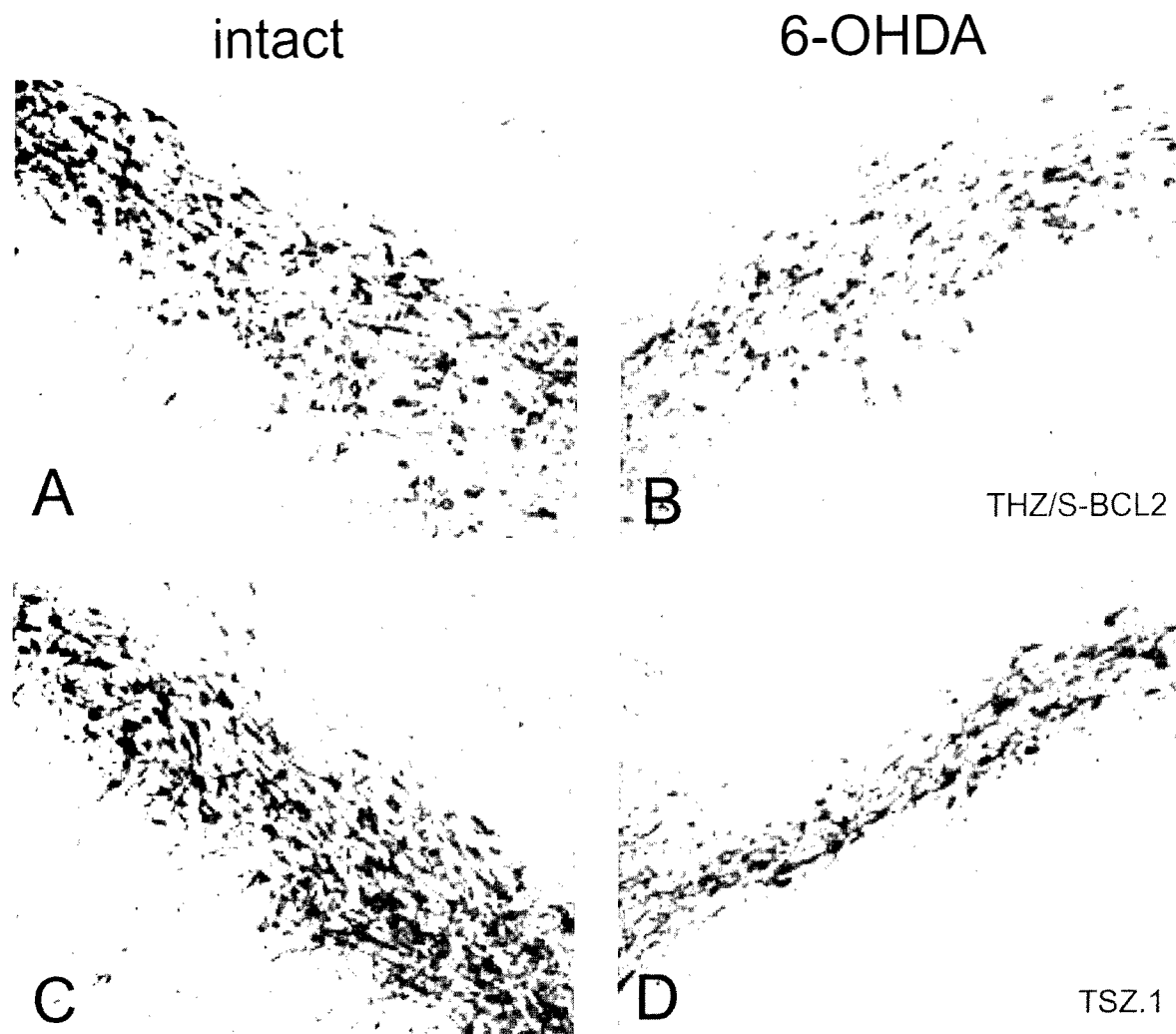


FIG. 7. Photomicrographs of intact and lesioned/vector-injected SN 2 weeks after 6 OHDA lesion (TH-immunoreactive cells). Representative photomicrographs of TH-immunoreactive cells in contralateral intact SN (A and C) and from ipsilateral SN injected with THZ/S-bcl2 (B) and TSZ.1 (D).

from degeneration induced by 6-OHDA. We have used the well characterized model described by Sauer and Oertel (6) in which nigrostriatal dopaminergic neurons degenerate over a period of weeks, with most of the cell loss completed by 2 weeks postlesioning. Retrograde labeling with FG demonstrates that the cell loss does not merely represent the loss of the dopaminergic phenotype of surviving cells; the amount of protection afforded was similar using either of the measures employed.

Evidence in cell culture model systems by others has demonstrated that exposure to 6-OHDA may result in cell death with features suggestive of apoptosis (11–13), and *in vivo* histologic and biochemical markers of apoptotic cell death can be demonstrated in the SN after 6-OHDA lesioning (10). Two recent reports have shown that transgenic mice overexpressing bcl-2 are resistant to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) toxicity (21, 26). Surprisingly, in the latter study, protection against the acute model of MPTP toxicity, which is characterized histologically by necrotic cell death, was more robust than protection against the “chronic” model of MPTP toxicity, in which cell death is more clearly apoptotic in nature (26).

It has been demonstrated previously that delivery of the glial-derived neurotrophic factor (GDNF) directly to the region of the SN (27) protects nigrostriatal DA cells from degeneration in the same model of 6-OHDA toxicity and that production of GDNF mediated by either an E1–E3-deleted

recombinant adenoviral vector (28, 29) or a recombinant adeno-associated viral vector (30) is also capable of protecting these neurons from degeneration and loss of TH immunoreactivity. The magnitude of protection demonstrated in our study, comparing control vector with the experimental vector, is similar to the magnitude of protection provided by the GDNF vectors. There is no direct evidence that GDNF effects are mediated through prevention of apoptosis. In cells of hematopoietic lineage, Bcl-2 delays ATP depletion induced by cytokine withdrawal (31). Whether the protective effect we see with Bcl-2 would be additive to the protective effects of GDNF remains to be determined. In addition to the effects of Bcl-2 on the caspase cascade in apoptosis, there is evidence that Bcl-2 may function directly to protect cells from injury because of oxidative stress (32–34). Bcl-2 shifts the redox potential to a more reduced state (35) and enhances mitochondrial calcium uptake (36). Some of these effects may occur upstream of caspase activation or independent of apoptotic mechanisms.

The HSV-based vector employed for transgene delivery and expression in this study has several potential advantages. Recombinant genomic HSV-based vectors have a large capacity (37–39), and during latency the viral genome is entirely, transcriptionally silent with the exception of the latency-associated transcript and transgene expression. The multiply deleted vector employed in this study is incapable of replicating in the absence of complementation of the essential immediate early genes ICP4 and ICP27 and, therefore, does not replicate

in vivo, and the relative absence of an immune response is a distinct advantage in the use of these vectors.

The 6-OHDA model is an excellent model of cell death restricted to dopaminergic neurons of the SN, although the relevance of the cell death in this model to that which occurs in human Parkinson's disease has not been established fully. Several investigators have reported detection of cells with morphologic characteristics of apoptosis in the SN of brains from Parkinson's patients examined at autopsy (40–43). The short time frame during which cells undergoing apoptotic cell death may be detected compared with the prolonged duration of the disease process makes large-scale detection of apoptotic cell bodies in the late stage of the disease unlikely. Nonetheless, if Bcl-2 expression can prevent such cell death in the human disease, long-term expression from a defective HSV vector may have therapeutic potential.

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MILLENNIUM REVIEW

Design and Application of HSV Vectors for Neuroprotection

DJ Fink¹, NA DeLuca², M Yamada³, DP Wolfe² and JC Glorioso²¹Departments of Neurology and Molecular Genetics and Biochemistry, University of Pittsburgh, School of Medicine and GRECC, VA Pittsburgh Healthcare System; ²Department of Molecular Genetics and Biochemistry, University of Pittsburgh, School of Medicine; and ³Departments of Neurology and Molecular Genetics and Biochemistry, University of Pittsburgh, School of Medicine Pittsburgh, PA, USA

Herpes simplex virus has been extensively genetically modified for gene transfer to nerve and other tissues, to create vectors that are devoid of viral gene expression and toxicity. Recombinant vectors have been engineered to express genes which protect neurons against toxic insults resulting in cell death, including nerve growth factor (NGF) and anti-

apoptotic genes (eg bcl-2). This review describes experiments using HSV vectors expressing these gene products and their potential protective role in ameliorating neurodegenerative processes in animal model systems. *Gene Therapy* (2000) 7, 000-000.

Keywords:

Herpes Simplex; Gene Transfer; Neurodegenerative Disease; Nerve Growth Factors;

Herpes virus is a naturally neurotropic virus that in natural infections shows a predilection for neurons of the peripheral nervous system. A unique aspect of the biology of HSV is its ability to establish a life-long latent state in neurons, latent genomes have no detectable effect on nerve cell function. Taking advantage of neural tropism and natural latency, we have developed a series of vectors designed to deliver and express genes in neurons for a variety of potential therapeutic applications.¹ The therapeutic gene product may be designed to function within the cell in which it is expressed, may be released from that cell to function locally either in an autocrine or paracrine fashion, or it may be released into the circulation to function systemically. This review will focus on recent studies showing that HSV-based vectors may be useful in the treatment of diseases characterized by neurodegeneration.

HSV is an enveloped double-stranded DNA virus with more than 80 identified genes encoded for by the 152 kb viral genome.² The virus envelope contains at least 10 glycoproteins which function in the attachment and penetration of the virus. Initial binding of the virus to cell surface glycosaminoglycans (GAGs), primarily heparan and dermatan sulfate is mediated by glycoproteins C (gC) and B (gB).^{3,4} This initial binding is followed by specific interactions between gD and identified receptors (HveM herpes virus entry mediators) HveA and HveC. HveA is a member of the TNF p75 receptor superfamily,⁵ but is generally thought not to be expressed in the nervous system. HveC belongs to the immunoglobulin superfamily and is highly expressed in the nervous sys-

tem.⁶ Sequential attachment to GAGs followed by specific interactions with HVEMs leads to fusion of the virus envelope with the cell surface membrane and entry of the viral capsid with its surrounding tegument proteins into the cell. The mechanism through which viral penetration into the cell occurs is not well understood, but requires multiple glycoproteins (gB, gH/gL and gD).

Following entry into a neuron the capsid is carried by retrograde axonal transport along the cytoskeleton to the nucleus, where the DNA is injected through nuclear pores to initiate the cascade of lytic gene expression. Wild-type viral genes are expressed in a temporally regulated cascade. Immediate early (IE) genes are expressed after viral entry into the nucleus and in the absence of *de novo* viral protein synthesis. IE proteins activate expression of the early (E) genes, and following E gene-mediated synthesis of viral DNA, the IE proteins promote the expression of late (L) viral genes. The IE gene products are largely regulatory proteins, the E gene products are involved in viral DNA synthesis, and the L gene products are largely structural proteins of the virion. The expression of IE genes is enhanced by VP16, a structural protein that acts in concert with several cellular transcription factors including Oct-1 to activate transcription of the IE gene promoters through recognition of one or more copies of the consensus element TAATGARAT. One IE gene product, ICP4, is absolutely essential for viral replication.^{7,8} Deletion mutants in ICP4 were the first HSV essential HSV gene knockout mutants to be constructed.⁹ An important consequence of deleting ICP4 from HSV is that the temporal cascade of viral gene expression is incapable of proceeding past the IE phase of transcription except in cell lines designed to provide ICP4 *in trans*. Thus, despite the complexity of the virus genome, the great majority of virus gene expression can be eliminated by a single deletion mutation.

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HSV

Correspondence: JC Glorioso, Department of Molecular Genetics and Biochemistry, University of Pittsburgh, School of Medicine, E1240 Biomedical Science Tower, Pittsburgh, PA 15261, USA

Although deletion of ICP4 is sufficient to create a vector that is incapable of replicating, the remaining IE gene products (ICP0, ICP22, ICP27 and ICP47) are abundantly expressed. The majority of these gene products can profoundly alter cell metabolism and gene expression and may be toxic to cells. Vector toxicity has been resolved by engineering virus mutants deleted for all the IE genes.^{10,11} A recombinant (d106) engineered to express only the IE protein ICP0 induces expression of a subset of cellular genes, while an isogenic recombinant (d109) which does not express any viral proteins had no appreciable effect on cellular gene expression.^{11,12} Thus the vectors deleted for multiple IE genes behave in a manner similar to UV-irradiated viral particles which are apathogenic to cells. These highly deleted vectors have several advantages. First, they can be propagated to high titers without the emergence of wild-type recombinants. Because the recombinant vector is propagated directly in complementing cells there is no helper virus. Second, the complete IE gene knockout vectors do not alter cell metabolism making them ideal for gene transfer to non-neuronal cells. Third, the expression of the LATs in neurons does not depend on expression of the IE genes making it possible to construct a highly defective vector that can readily persist in a latent-like state in neurons and transgenes can be expressed using the latency promoter.

In wild-type infection the virus either replicates, or alternatively enters a latent state, in which the genomes exist as circular or concatemeric intranuclear episomes in which the lytic functions are transcriptionally silent. It has been known for some time that viruses prevented from replicating are forced into the equivalent of latency.¹³ Similarly, recombinant vectors deleted for one or more essential IE genes can establish a persistent state in which the lytic genes are essentially silent and the state of the genome may be similar to that of natural latency. In this state a single region of the latent genome continues to be transcribed producing a family of non-polyadenylated latency associated transcripts (LATs), which persist as intranuclear RNAs known to be stable intron lariates.^{14,15} Although the function of LATs is not known, their production (which may persist for the life of the host) serves as the hallmark of latent genomes in the nervous system and demonstrates that sequences may be transcribed from the latent genomes. It should be noted that only a fraction of latently infected cells express LATs at a level detectable by *in situ* hybridization (ISH), so that the demonstration of LATs by ISH under-represents the total number of latently infected neurons.^{16,17}

In recent studies we have found that HSV-based vectors deleted for multiple IE genes can be used to deliver neuroprotective macromolecules to neurons. Two different paradigms have been employed. Nerve growth factor (NGF) is the prototypical neurotrophic factor, originally identified by its ability to protect peripheral sensory and sympathetic neurons from programmed cell death during development. In addition to its action during development, NGF is able to protect adult sensory and sympathetic neurons against a variety of insults that include: axotomy, ionophore treatment, exposure to hydrogen peroxide, or excitatory amino acids. In animal models, systemic administration of high doses of NGF prevents the onset of peripheral neuropathy after toxin exposure or after the induction of diabetes by streptozotocin. The bioactivity of the 118 amino acid β subunit can be

assayed *in vitro* by its effect in inducing neuronal differentiation and neurite sprouting in PC-12 cells.

Genomic HSV-based vectors containing the coding sequence for β -NGF under the transcriptional control of either the transiently active human cytomegalovirus immediate-early promoter (HCMV IEp) element, or a HSV latency active promoter (LAP2), produced biologically active NGF as measured by the differentiation and neurite sprouting of transfected PC-12 cells.¹⁸ NGF expression in trigeminal ganglia *in vivo*, following topical corneal infection and subsequent retrograde axonal transport, was high 3 days after inoculation with the HCMV IEp:NGF vector but fell by 21 days.¹⁸ In contrast, the LAP2:NGF vector showed a low level of expression at 3 days but a high level at 21 days, consistent with the known kinetics of that promoter element. Vector-mediated expression of NGF in neurons of the dorsal root ganglion *in vitro* was consistent with expression in trigeminal neurons *in vivo* with the HCMV IE promoter displaying transient activity whereas LAP2 remained active at 2 weeks (Figure 1). Vector-mediated NGF synthesis induced expression of superoxide dismutase and catalase, and was effective in protecting these cells from apoptosis induced by exposure to hydrogen peroxide (Figure 1).¹⁸ This effect also demonstrated kinetics consistent with the known promoter activity, higher early with the HCMV IE promoter and at later times with the LAP2 element (Figure 1).

NGF activity is mediated by cell surface receptors

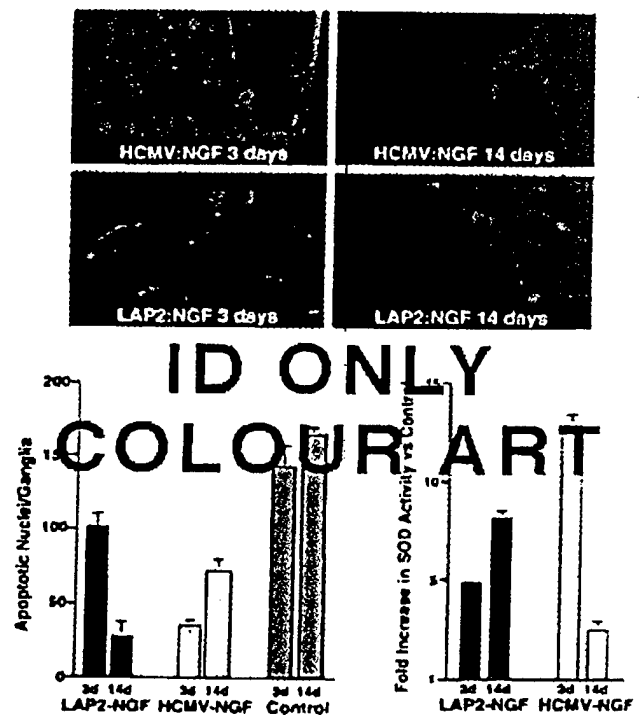


Figure 1 HSV vector-mediated NGF expression protects DRG neurons from toxic insult. DRG neuronal cultures infected with the HCMV:NGF vector express high levels of NGF at 3 days that protects DRG neurons from apoptotic insult by increasing the levels of antioxidant enzymes such as SOD. In contrast, LAP2 driven NGF expression at 14 days blocks apoptotic insult and results in increased SOD enzyme activity.

(trkA and p75) which once activated by NGF binding to the extracellular domain of the receptor initiates a cascade of intracellular signals beginning with dimerization and autophosphorylation of the intracellular domain of the trkA, leading to phosphorylation of PLC, PI-3 β , and the adaptor protein Shc, and ultimately activation of a MAP kinase cascade. Because these actions are initiated by binding of NGF to the extracellular domain of the receptor, we presume that transgene-coded NGF must be released from transfected cells to bind to receptors on those and neighboring cells to produce the biological effect. This mode of action allows amplification of the biological effect achieved by release of trophic factors from the transduced cells.

The endocrine effect of transgene-produced NGF has been demonstrated systemically in rabbits and monkeys. Animals were inoculated into one knee joint with replication-defective HSV vectors containing the coding sequence for β -NGF under the control of either the LAP2 or HCMV:IEp promoter. Both vectors expressed NGF that could be detected in the joint space and in the plasma reached levels in blood of 30 ng/ml within weeks of vectors administration.¹⁹ Expression persisted for more than 1 year. While the level of NGF in blood could be accounted for by the amount of injected virus, it appeared more likely that an autocrine loop driven by vector expression was responsible for the high levels observed. The released NGF induced the sprouting of the terminals of peripheral nerve in the skin, quantitated by direct counting of biopsied material (Figure 2).

Expression of trophic factors represents one potential approach to protecting neurons from a variety of insults or degenerative conditions. Alternatively, for those conditions in which neuronal cell death proceeds through apoptotic pathways, HSV-based vectors can be used to interrupt the apoptotic pathway at downstream sites through transgene-driven expression of anti-apoptotic peptides such as bcl-2. Bcl-2, a structural and functional homologue of the nematode protein CED-9 prevents the opening of the permeability transition complex of mitochondria and the subsequent release of cytochrome C among other functions. Bcl-2 is located on the cytoplasmic face of the mitochondrial outer membrane, the endoplasmic reticulum, and the nuclear envelope, and acts within the cell in which it is produced. We have studied a bcl-2 expressing vector in two model systems.

The neurotoxin 6-hydroxydopamine (6-OHDA) is widely used to model degeneration of dopaminergic neurons of the substantia nigra characteristic of Parkinson's disease. Injection of the toxin into striatum causes a subacute lesion with a well-defined morphologic, behavioral, and biochemical phenotype. p53 and Bax protein expression are up-regulated by exposure of cells to 6-OHDA, and PC-12 cells exposed to 6-OHDA demonstrate cell shrinkage, chromatin condensation, membrane blebbing and DNA fragmentation characteristic of apoptosis. Dopamine-induced apoptosis of PC-12 cells is inhibited by overexpression of bcl-2, and cultured neurons from bcl-2 overexpressing transgenic mice are resistant to 6-OHDA toxicity *in vitro*. We therefore tested whether transgene-driven expression of bcl-2 could prevent degeneration of substantia nigra neurons characteristic of Parkinson's.

The human bcl-2 cDNA under the transcriptional control of the simian cytomegalovirus (SCMV) IEp was

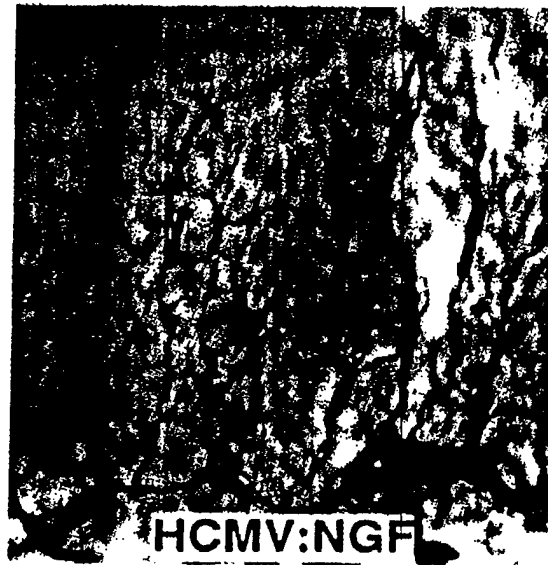
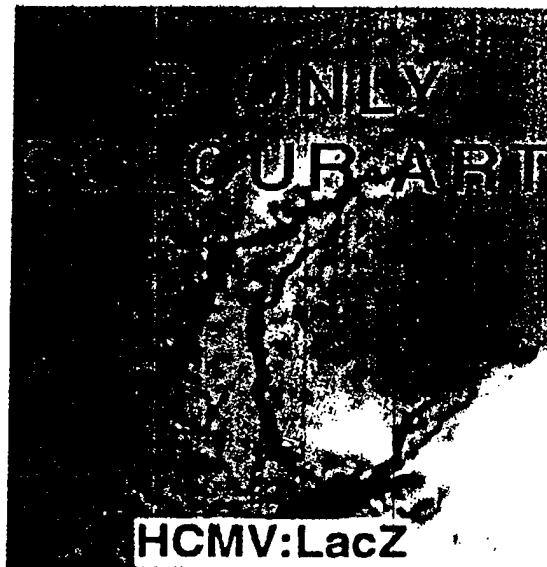


Figure 2 A vector expressing NGF from the HCMV promoter increases neurite density in peripheral skin biopsies. The number of neuron terminals was blindly counted in samples taken from control, lacZ expressing vector, or NGF expressing vector inoculated monkeys. Approximately twice as many nerve endings are evident 5 months after NGF vector administration.

placed in the thymidine kinase (tk) locus of a genomic HSV vector deleted for the HSV IE genes ICP4, ICP22 and ICP27.²⁰ This vector produced immunoreactive bcl-2 in cultured cells, and protected cortical neurons *in vitro* from naturally occurring programmed cell death. The bcl-2 expression vector (or the control lacZ expressing vector) was injected directly into the substantia nigra unilaterally; at the same time fluorogold was injected into the striatum bilaterally in order to label neuronal cell bodies in the substantia nigra that project to the striatum. One week later 6-OHDA was injected unilaterally into

striatum, using the same coordinates as for fluorogold. Two weeks after 6-OHDA injection the animals were killed and the survival of FG-labeled nigral neurons and tyrosine hydroxylase (TH) immunoreactivity of the lesioned substantia nigra compared between bcl-2 vector-injected and control vector injected animals.²⁰

Bcl-2 produced from the vector prevented 6-OHDA induced degeneration of retrogradely labeled FG-positive neurons, and also preserved the TH immunoreactive phenotype of the lesioned neurons (Figure 3). Approximately twice as many cells of the SN, labeled retrogradely by FG or identified by TH immunoreactivity survived in the bcl-2 vector-treated animals compared with control vector-injected animals.²⁰ The magnitude of the protective effect of vector-mediated bcl-2 expression was similar to that reported in bcl-2 over-expressing mice exposed to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) suggesting that a substantial proportion of the at-risk cells were protected by vector-mediated transgene production. Because bcl-2 is located and acts intracellularly, the neuroprotective effect would be expected to occur only in transfected cells.

It has not been established whether the degeneration of dopaminergic neurons in Parkinson's disease is correctly modeled by 6-OHDA lesioning and proceeds through apoptotic pathways. We have recently begun to explore the utility of this vector in preventing cell death in another model that is directly relevant to human disease. The peripheral nerve is relatively resistant to trauma, and peripheral axons demonstrate a remarkable ability to regenerate following injury. However, after proximal root avulsion, motor neurons in the ventral horn of spinal cord degenerate rapidly, and even prompt surgical anastomosis of the proximal stump to the distal segment fails to promote regeneration and restoration of function. It is not known whether motor neuron death following proximal root avulsion is apoptotic, but we reasoned that if it were, the bcl-2-expressing vector might protect those cells from immediate death and allow for recovery and ultimately regeneration.

The experimental model is analogous to that employed in the 6-OHDA model. The bcl-2 expressing vector or a

control vector was stereotactically injected unilaterally into the ventral horn of lumbar spinal cord. At the same time, fluorogold was injected into the sciatic nerve bilaterally in order to label motor neurons projecting into the nerve. One week later the roots were exposed and surgically avulsed. After 2 weeks survival the rats were killed. The number of surviving motor neurons determined by analysis of the fluorogold labeling demonstrated a substantial and statistically significant protective effect of vector-mediated bcl-2 expression. In contrast to the 6-OHDA lesioning experiment, bcl-2 expression did not preserve the neurotransmitter phenotype of the injured cells. Immunostaining for choline acetyltransferase (ChAT) revealed an almost complete loss of ChAT immunoreactivity in bcl-2 vector, control vector, or PBS-injected lesioned animals. The loss of cholinergic phenotype may result from the difference between the two lesions (terminal damage in 6-OHDA *versus* proximal damage in the spinal cord model), a difference between central and peripheral neurons, or the requirement for other factors to support the neurotransmitter expression in surviving neurons. To that end, we are now investigating whether surgical repair of the nerve allows regeneration and repair in the bcl-2 treated animals, and whether a vector expressing both the anti-apoptotic peptide and a trophic factor might be more effective than either alone.

⑤ The studies reviewed above demonstrate that it is feasible to protect neurons from a variety of insults using a genomic HSV-based vector expressing either neurotrophic factors or anti-apoptotic peptides. Transgene expression may be targeted to specific sites in the central nervous system by stereotactic inoculation to cells in the peripheral nervous system by retrograde axonal transport from the periphery, or released to function systemically in an endocrine manner. The choice of trophic factor and antiapoptotic peptide for specific applications will need to be determined empirically, but capacity of the HSV vector makes it possible to express several transgenes simultaneously from a single vector should that prove necessary.

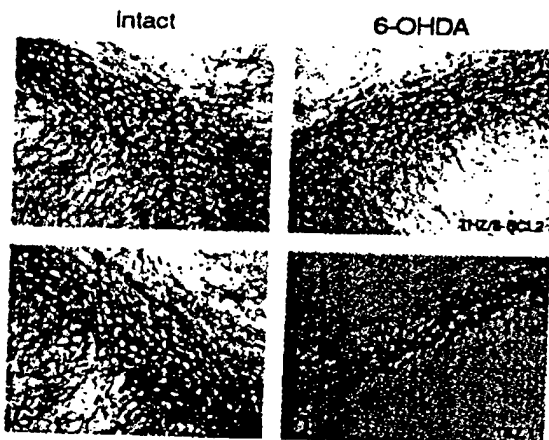
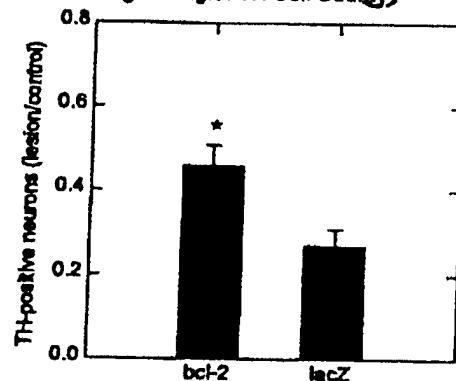


Figure 3 A bcl-2 expressing vector protects neurons from 6-OHDA toxicity. The number of surviving TH-immunoreactive neurons in bcl-2 expressing vector-injected SN is significantly greater than in control vector injected SN. (Reprinted with permission from PNAS 96(7): 4078-4083. Copyright (1999) National Academy of Sciences, USA).

the transgene product may function locally (Nigral TH Cell Count)



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